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14. ABSTRACT The effort explores the preparation theranostics comprising a therapeutic peptide and a chelating group based on DOTA. Methods to develop a series of dendrimers bearing an imaging group at the core evolved from late-stage alkyne functionalization to early incorporation of the imaging group. Efforts showed that dendrimers of varying sizes can be accessed with this strategy. Methods for the accelerated synthesis of these targets were developed. Solubility parameters were also defined. Methods to incorporate multiple copies of a therapeutic peptide rested on the utilization of linkers that were either biolabile or stable. Efforts with biolabile linkers revealed a new class of hydrazones that show promise in multiple areas of therapeutic research, although more limited utility to date with highly soluble cargos (the peptide of interest). Efforts with stable linkers included maleimide and therapeutics bearing a thiol (cysteine) and covalent ligation resulting from selective triazine addition of a functionalized therapeutic. The latter strategy is favored based on the instability of the maleimide and the need for long reaction times.					
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INTRODUCTION

The long term goal of the proposed project was to explore novel theranostics to stifle metastasis of prostate cancer. The sub-award described here focused on the preparation of these labeled, multivalent displays of peptides in an effort that proceeded through five phases.

Phase 1 (Projected 0-6 months): The goal of phase 1 efforts was to prepare dendrimers ranging from generation 3 to 7 with an alkyne group at the core which could be subsequently functionalized with a chelate for diagnostic imaging. The projected time for this initial synthetic effort was 6 month.

Phase 2 (Projected 4-12 months): An additional 6 months was predicted to be required for developing methods for the installation of the chelate using alkyne-azide click chemistry.

Phase 3 (Projected 9-18 months): The third phase aimed to probe the solubility characteristics of the dendrimers by manipulating the surface groups. These activities were hypothesized to be completed by the 18 month mark.

Phase 4 (Projected 12-24 months): Strategies for the design and incorporation of therapeutic peptides into dendrimers were expected to proceed over the next year with submission of candidates to UTSW by the end of year two. The initial candidates were low-valency targets presenting up to 8 therapeutic peptides.

Phase 5 (24-48 months): An iterative strategy for optimizing activity based on leads was the goal of these efforts.

KEYWORDS

theranostic, dendrimer, triazine, peptide, synthesis, linker, microwave synthesis, maleimide, hydrazone, release, imaging, DOTA, chelation

ACCOMPLISHMENTS

The proposed statement of work identified four action items shown in table 1 and a fifth goal of refinement of activity. These goals and the degree of their completion are indicated.

Table 1. Summary of Major Goals

Task	SOW Description	% Done
1	Synthesis and characterization of dendrimers that vary in size from generation 3-7 with a functional alkyne	100%
2	Installation of a chelate group for diagnostic medical imaging ^a	100%
3	Exploration of surface groups to promote desired behavior ^b	100%
4	Therapeutic peptides will be designed and installed	100%
5	Iteration to yield an active target	failed ^c

^aA change from a "synthetically expensive" chelate to an inexpensive and commercially/clinically relevant alternative was undertaken when the synthetic effort required earlier installation of this group than was originally anticipated. The shift was deemed insignificant given the SOW.

^bSurface groups were initially believed to be the greatest contributor to physicochemical properties, but research showed the the interior groups played a much more dominant role in determining solubility and computational models in related systems reinforced this hypothesis and observation. The shift was deemed insignificant given the SOW.

^cMultivalent displays failed to reproduce the activity of the monomeric peptide initially reported. A redesigned monomer showed reduced activity and was synthetically more difficult to obtain.

While the long term goal of a multivalent, bioactive theranostic was achieved, a number of major accomplishments can be identified that stemmed from this work. These accomplishments appear in Table 2. The status of dissemination is also indicated.

Table 2. Summary of Major Accomplishments

Task	Accomplishment	Dissemination Status
1	Synthesis of G3-G6 dendrimers with an alkyne core	Published
1	Evaluation of click-chemistry within triazine dendrimers	Negative result
1	Rapid, microwave synthesis efforts of dendrimers	Published
NA	Using intrinsic fluorescence to probe structure	Under review
2	Synthetic strategies for carrying a chelate through multi-step dendrimer synthesis relying on orthogonal deprotection of acid labile groups	Published
3	Structure-based understanding of dendrimer solubility in water as a function of interior (not surface!) groups	Published
4	Scope and limitations of maleimide-terminated dendrimers for ligand/peptide display	Published
4	Scope and limitations of covalent ligation of peptides bearing cyclic amines to triazine dendrimers	Negative result
4	Investigation of a novel triazinyl hydrazine and hydrazone formation for biolabile displays of ligands	Published
NA	Triazinylhydrazines show selective conjugation to drugs	In preparation
4	Scope and limitations of ligation of peptides bearing an aldehyde or ketone group with dendrimers displaying multiple triazinylhydrazines	In preparation

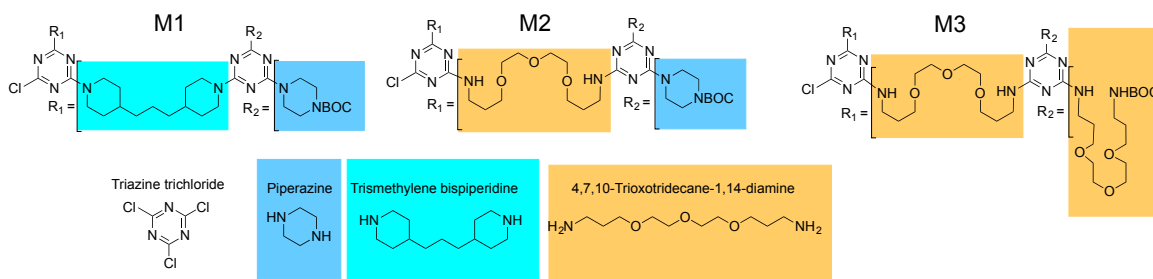
The long term goal of delivering biological activity within a multivalent theranostic was not met. However, the list of major accomplishments serve to reinforce the value of triazine dendrimers as candidate platforms for nanomedicines based on increasing ease of synthesis, an understanding of how interior groups control the solubility properties of these architectures, and the operational space available for ligating groups of interest (here the therapeutic peptide and models) using covalent and biolabile linking groups.

Many of these accomplishments have been published in the scientific literature, and these papers appear in the appendix. The following paragraphs summarize those results that have not been published. The following paragraphs provide details for each task of the SOW.

Task 1: Synthesis and characterization of dendrimers that vary in size from generation 3-7 with a functional alkyne

Dendrimer synthesis relies on iterative, polymer chemistry. The strategy is most efficient when common building blocks are readily available. For this task, three building blocks were prepared (Chart 1). These vary by the choice of linking diamine (shown in a colored box). Each macromonomer (**M1-M3**) provide two generations of dendrimer for every reaction.

Chart 1. Macromonomers **M1-M3** with the linking diamines indicated with color.



To obtain the desired dendrimers (Chart 2, following page), a macromonomer is reacted with propargyl amine to afford the alkyne core. Then, the peripheral BOC-protected amines are unmasked and the resulting material is reacted with macromonomer. The process of deprotection and reaction is iterated until the desired product results. An important lesson emerged from the library of molecules shown in Chart 2. Water solubility of a dendrimer with a hydrophobic core (derived from **M1**) could be rescued if **M3** was used on the periphery. Surprisingly, the hydrophilic, cationic character of **M2** was insufficient.

Task 2: Installation of a chelate group for diagnostic medical imaging

The initial SOW called for manipulation of the single alkyne installed at the core of the dendrimer using click chemistry so that any imaging agent might be installed, although we proposed an experimental chelator of copper from co-PI Xiankai Sun's lab as the focus of these efforts. Preliminary model studies of copper-catalyzed click reactions failed to reproduce the levels of conversions reported in the literature, rarely exceeding 25% in unhindered, low-generation dendrimers. Computer simulations that suggested that the linking groups that were required to promote water solubility also were likely to shield this alkyne from reaction. (The more rigid, hydrophobic core of the dendrimer was intended to promote access of the click reagents.)

These failures led to a shift in strategy wherein the imaging group was installed early in the synthesis. To mimic the experimental chelator, a much less expensive, commercially available, conventional DOTA group was used. The goal was to optimizing bioactivity before optimizing the imaging. The strategy required the development of methods that allowed for the deprotection of peripheral BOC-groups on the dendrimer while retaining the protective t-butyl esters of the DOTA group. Conditions were uncovered that worked surprisingly well. The synthetic plan that was adopted is shown in Scheme 1.

Task 3: Exploration of surface groups to promote desired behavior

This task was completed given the solubility promoted by the groups discovered in pursuit of Task 1.

Chart 1. Targets of macromonomer synthesis.

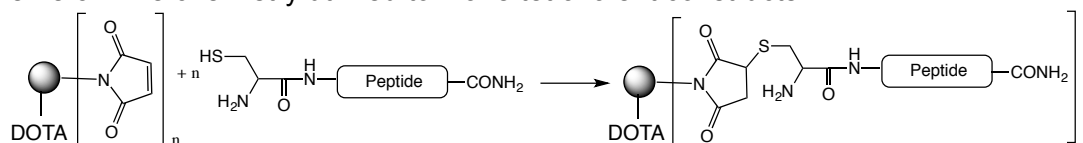
Compound	Organic Solubility	Aqueous Solubility	Organic Solubility (Protected)	
1	Y	N	Y	
2	Y	N	Y	
3	Y	Low <3mg/mL	Y	
4	Y	High 20mg/mL	Y	
5	N	N	N	
6	N	Very Low <<1mg/mL	Y	
7	Y	Low <1mg/mL	Y	
8	Y	Very High >50mg/mL	Y	

proline, but when an azetidine is incorporated during solid phase synthesis, acid-catalyzed cleavage from the resin destroys the ring. For *N*-terminal installation of an azetidine, a base-labile Fmoc-azetidine acid and soluble peptide would be required, or an alternative to the standard Wang solid phase resin. Incorporating this azetidine on a native peptide would prove challenging in the presence of competing lysine sidechains. Azetidine degradation can be avoided if the C-terminus is targeted instead, with aminoazetide. Similarly, aminopyrrolidine and aminopiperazines are now hypothesized to accomplish similar ends. However, the lessons learned from this chemistry are now being considered for use in the creation of new therapeutics, including next-generation cyclic antibiotics based on nisin which, serendipitously, was reported last year to show anticancer activity in rats.

Strategy 4.2: Non-labile attachment via maleimide chemistry

The use of maleimide chemistry developed in the laboratory was explored for the therapeutic peptide of interest. The chemistry relied on synthesizing dendrimers with maleimide groups on the periphery and reacting them with the therapeutic peptide containing an additional *N*-terminal cysteine (Scheme 3). The constructs resulting from this reaction are not ordinarily considered "biolabile", but the perceived ease of access, storage, and hypothesized stability *in vivo* led us to pursue them briefly. The maleimide dendrimer prepared featured the DOTA group at the core. The constructs derived showed no biological activity.

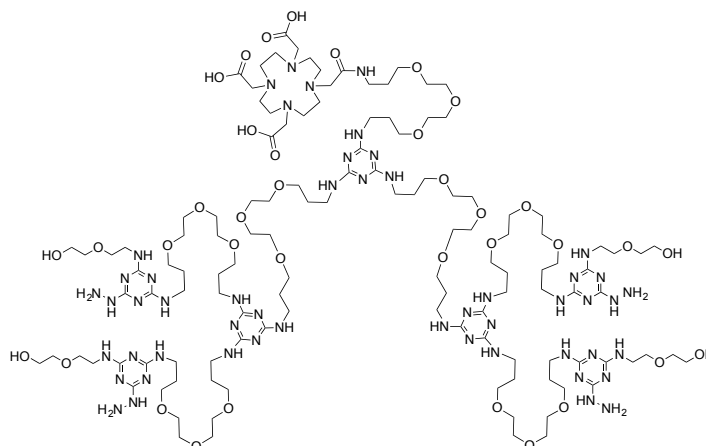
Scheme 3. The chemistry utilized to make tetravalent constructs.



Strategy 4.3: Biolabile linkage derived from triazinyl hydrazines

The lack of activity seen in multivalent constructs that displayed peptides using non-labile covalent bonds led to the exploration of hydrolytically sensitive linkages. The chemistry explored presented an extension of the triazine chemistry of interest. Triazines bearing hydrazine groups were prepared as acylhydrazine equivalents. The work showed that triazinylhydrazine condense with the carbonyl groups of aldehydes and ketones. The intrinsic advantage of using a triazine comes with the potential to rapidly elaborate it to a theranostic with suitable biophysical properties. We reported the stability and kinetics of release of model systems wherein the triazinylhydrazine showed higher stability than the corresponding acylhydrazones at pHs below the pK_a of the triazine. Whether this can be exploited for these intents, there is potential applications of these materials for buffering in gene therapy applications. The triazinylhydrazine chemistry that has been reported has been elaborated to prepare dendrimers displaying multiple groups as shown in Chart 2. The compound reacts efficiently with simple aldehydes and ketones, and with bioactives including the drug doxorubicin and bruceantin. Peptides bearing an *N*-terminal *p*-formylbenzoic acid group have been explored.

Chart 2. A theranostic platform comprising a DOTA imaging chelate and four triazinyl hydrazines.



Task 5: Iteration to yield an active target

Throughout the efforts, the only target that proved to have activity competitive with the lead construct was a monomer that presented a triazine linker between the therapeutic peptide and targeting sequence derived from an arginine-rich sequence. Studies of this peptide are ongoing.

Training

The project served as a mechanism for professional development for the post-doctoral fellow who executed early efforts, the graduate student who took over as his skill set developed, an international research exchange student who explored elements of these chemistries (specifically, polyproline synthesis and reactivity), and simultaneously catalyzed a cross department collaborative effort. Neither the research exchange student nor departmental collaborators (Professors Janesko, Minter, and more recently Green) derived any financial support from the project, but became engaged in the different elements (computation, hydrazone experimental efforts, and metal coordination, respectively).

Dissemination has centered on the submission and publication of peer-reviewed manuscripts. Six manuscripts have appeared in print and two more will be submitted in the upcoming months. An additional 2-4 that acknowledge this grant are expected in the 2017-2018 period. Table 3 summarizes this activity.

In addition, manuscripts describing 1) the ability of triazinyl-N-methyl-hydrazines to condense with carbonyls to form hydrazones and 2) the biological activity of some hydrazones are expected in the next year. A full account of the last fifteen years worth of exploration is being outlined for *Accounts of Chemical Research*.

Table 3. Products of dissemination currently identifiable

#	Title	Status
1	Influence of linker groups on the solubility of triazine dendrimers	<i>New J. Chem.</i> 2015
2	Accelerated synthesis of large generation triazine dendrimers using microwave assisted reactions: a 24 hour challenge	<i>Polymer Chem.</i> 2015
3	Triazine-Substituted and Acyl Hydrazones: Experiment and Computation Reveal a Stability Inversion at Low pH	<i>Molec. Pharm.</i> 2015
4	Functionalization of a triazine dendrimer presenting four maleimides on the periphery and a DOTA group at the core	<i>Molecules</i> 2016
5	Thermoregulated Coacervation, Metal-encapsulation and Nanoparticle Synthesis in Novel Triazine Dendrimers	<i>Molecules</i> 2016
6	Intrinsic Fluorescence of Triazine Dendrimers Provides a New Approach to Study Dendrimer Structure and Conformational Dynamics	<i>J. Phys. Chem. C</i> 2016
7	Triazine-substituted hydroxypyrazoles: Synthesis and metal binding studies	For <i>Inorg. Chem.</i>
8	Functionalization of a triazine dendrimer displaying hydrazine-substituted triazines on the periphery	For <i>Canadian J. Chem</i>

IMPACT

On the discipline. The primary impact on the specific discipline of polymer chemistry, more specifically, dendrimer chemistry, was to conquer remaining challenges including the identification of methods for the rapid synthesis of these materials and strategies for conjugating both diagnostic groups and multiple bioactives.

On other disciplines. The lessons learned make this platform increasingly useful for clinical future clinical relevance. One specific example came to light in mid-2016 when a biotech company (in collaboration with the NIH) identified the triazine chemistry developed here as motivation for pursuing explorations of a platform for gene therapy. Collaborative efforts are commence next month. In another example, the ability of the triazinyldiazines to coordinate metals suggest a role in combatting cancer and other diseases where oxidative stress can be exploited. Collaboration with Dr. Kayla Green in our department are proceeding. The application of this chemistry to the creation of new bioactives with potential anti-cancer and anti-microbial activity is also expected.

On tech transfer. While no patents have been issued covering this work, the lessons learned can be applied (and are being pursued) in areas where intellectual property is expected to be generated as described above.

On society. While limited now to the department and college, the effort has initiated broad interest in focused research collaboratives including theranostic nanomaterials and antimicrobials. The ability to attract international student researchers to work on these projects the US has untold implications.

CHANGES/PROBLEMS

No substantive changes in delivery of the SOW were encountered that extended beyond what is broadly considered scientific exploration and optimization. We are left with a significant problem outside our foreseeable control: it is unlikely that multivalent displays of a peptide envisioned to interact with the intracellular target of interest is likely to yield advantageous activity that overcomes the burdens of synthesis and characterization associated with its creation. Multivalent displays have shown promise with extracellular receptors. However, this "problem" served to focus synthetic efforts that led to major accomplishments.

PRODUCTS

The products, research papers, were enumerated in Table 3.

PARTICIPANTS

The participants supported financially on this project included:

Dr. Changsuk Lee, postdoc, 32 person months, who initiated these efforts with emphasis on the maleimide chemistry and creation of the theranostic platform.

Mr. (now Dr.) Kun Ji, graduate student, 16 person months, who took over the project from Dr. Lee, developed the hydrazone chemistry, and explored their use in dendrimers and with novel bioactives including bruceantin.

Participants that worked on this project, but were not supported financially included:

Mr. (now Dr.) Alan Enciso, graduate student, 48 months, who developed the rapid synthesis of the dendrimers and explored solubility properties as a function of internal linker.

The Fluorescence subgroup including Mr. Enciso, Mr. Akop Yepremyan, and members of Dr. Karol Gryczynski's photophysics group who explored the intrinsic fluorescence of the materials prepared by Mr. Enciso and Yepremyan.

Mr. Vishal Sharma and Mr. Akop Yepremyan who are extending the chemistry of the triazinyldiazines.

Mr. Fermin Ramirez-Crescencio and Mr. Benjamin Large, international research exchange students who assisted Mr. Enciso in various aspects of his studies.

Professors Benjamin Janesko, Onofrio Annunziata, David Minter, and Kayla Green all of this department who collaborated on computation, biophysical characterization, synthetic and spectroscopic characterization, and metal coordination respectively.



Cite this: *New J. Chem.*, 2015, 39, 1247

Influence of linker groups on the solubility of triazine dendrimers†

Alan E. Enciso,^a Matteo Garzoni,^b Giovanni M. Pavan^b and Eric E. Simanek^{*a}

Eight triazine dendrimers were prepared to probe the impact of linker choice on water solubility. Three different linkers were assessed including two hydrophobic diamines that show high reactivity, piperazine and trimethylene bispiperidine, as well as a hydrophilic diamine, 4,7,10-trioxotridecane-1,14-diamine, which is less reactive. Dendrimers **1–8** share a common, generation two, hydrophobic core, **1**. Dendrimer **1** is insoluble in water. Of the three generation four dendrimers, **2–4**, that were prepared, **2** is also insoluble in water, but substitution of one or two of the hydrophobic linkers with 4,7,10-trioxotridecane-1,14-diamine yields sparingly soluble **3** and more soluble **4**, respectively. Molecular dynamics simulations of dendrimers **2–4** in water provide additional insight into their shape, hydration and hydrophobicity. Generation six targets, **5–8**, are also sensitive to choice of interior and surface groups. Dendrimer **5** is insoluble in water, but replacing one or two hydrophobic linkers with 4,7,10-trioxotridecane-1,14-diamine yields dendrimers **6** and **7** with modest affect unless the double substitution occurs in tandem at the periphery to yield **8** which shows high solubility in water. The solubility trends suggest that the choice of cationic surface group is critical, and that piperazine groups on the periphery and interior do little to promote solubility of triazine dendrimers in water compared with the hydrophilic amine 4,7,10-trioxotridecane-1,14-diamine.

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Introduction

Dendrimers are often considered for applications that require solubility in aqueous solutions including their use as drugs and drug delivery vehicles.¹ Solubility behavior is most commonly attributed to the nature of the surface groups which can be chosen to manipulate the type and density of charge or other solubilizing groups like poly(ethyleneglycol).² In addition to the periphery, the interior groups of triazine dendrimers are subject to facile manipulation because the dendrimers comprise triazine branching points and linking diamines.³ Historically, the selection criteria for the diamine—while influenced by whether a convergent or divergent approach was being adopted—rested on (i) the reactivity of the diamine, (ii) its cost, and (iii) the commercial availability of protected derivatives. Given the wealth of diamines that meet many of these expectations, the choice of diamine incorporated into triazine dendrimers has evolved over time.

Initially, *p*-aminobenzylamine was favored because the difference in the relative reactivity of the individual amines offered an

opportunity to execute a convergent syntheses without functional group interconversions or protecting group manipulations.⁴ However, reactivity was sluggish in comparison to other choices like 4-aminomethylpiperidine and intermediates containing the former discolored over time.⁵ Piperazine became a linker of choice for small dendrimers due to its high reactivity (compared with primary amines) and the commercial availability of a low cost BOC-derivative.⁶ Unfortunately, synthesis was limited to generation 3 targets due to solubility limitations attributed to the disc-like shape of the molecules.⁷ To preserve the reactivity of constrained diamines and introduce flexibility, trimethylene bispiperidine was explored. The dendrimers that resulted displayed higher solubility than all-piperazine molecules, but access to high generation materials was still limited based on solubility.⁸

More recently, 4,7,10-trioxotridecane-1,14-diamine has been employed (Chart 1). Here, we probe the role of combinations of these different amines on the solubility of the resulting dendrimers. Chart 2 shows the targets in this phenomenological

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† Electronic supplementary information (ESI) available: Complete experimental procedures, spectral data, and schemes. Figures associated with computation. See DOI: 10.1039/c4nj00917g

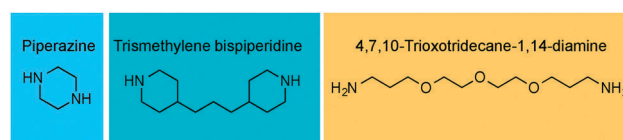


Chart 1 Diamines employed in this study.

Chart 2 Dendrimers examined in this study. The organic and aqueous solubility data are shown in columns 2 and 3. Column 4 reports the solubility of the BOC-protected precursor in organic solvent.

study. Sharing a common hydrophobic core, triazine dendrimers **1–8** were chosen to anchor our intuition on both (i) the nature of the cationic peripheral group and (ii) the influence of piperazine groups in conveying solubility.

Results and discussion

All targets examined in this study derive from a hydrophobic interior with modest flexibility. This generation 2 dendrimer, **1**, displays 8 piperazine groups on the surface and an alkyne at the core. Dendrimer **1** can be elaborated with macromonomers **9–11** shown in Chart 3 to yield the compounds **2–8**. Specifically, **1** is reacted with **9**, **10**, or **11** to yield BOC-protected derivatives of **2**, **3**, or **4**, respectively. Deprotection with a 1 : 1 mixture of MeOH and conc., HCl yields **2**, **3**, or **4**. Dendrimers **2** and **3** are further reacted with **9**, **10**, or **11** to yield protected **5–8** which are similarly deprotected. Solubility tests were performed by addition of dendrimer to one milliliter of Millipore water (18 MΩ cm). Sonication was used to determine saturation as measured with the naked eye. The solubility observations are reported in Chart 2.

The data anchors our intuition about solubility in three ways. First, substitution of the flexible hydrophilic linker 4,7,10-trioxotridecane-1,14-diamine for hydrophobic trismethylene bispiperidine within the interior of the dendrimer conveys critical, albeit slight water solubility to dendrimers. That is, **2** is insoluble while **3** is sparingly soluble. Similarly, **5** is insoluble while **6** and **7** show increased solubility. Solubility increases from $5 < 6 < 7$ as do the number of substitutions of 4,7,10-trioxotridecane-1,14-diamine for trismethylene bispiperidine. Second, the nature of the cationic peripheral group appears to have a profound affect on solubility. That is, replacing the terminal piperazine group with 4,7,10-trioxotridecane-1,14-diamine at the dendrimer surface significantly increases solubility. Dendrimer **4** is much more soluble than either **2** or **3**. Similarly, dendrimer **8** is much more soluble than **5**, **6**, or **7**. Third, the trends are conserved across all generations. Dendrimers **1**, **2** and **5**

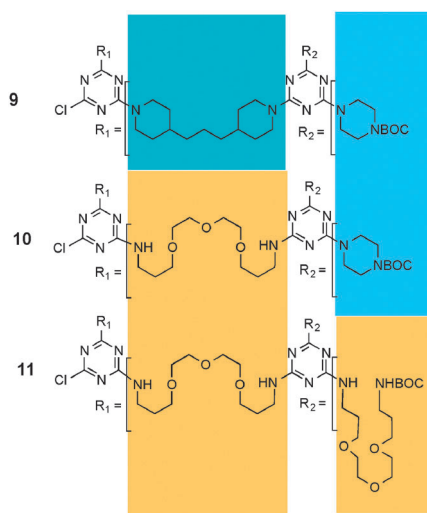


Chart 3 Macromonomers used in the synthesis.

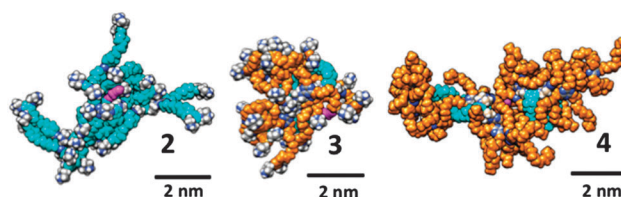


Fig. 1 MD simulation of **2–4** dendrimers in solution. Equilibrated (last) snapshot taken from the MD simulations of **2**, **3** and **4**. Trismethylene bispiperidine and 4,7,10-trioxotridecane-1,14-diamine are colored in cyan and orange respectively. The central residue of the dendrimers is colored in pink. Piperazine and all other groups are colored per-atom (C: grey, N: blue, H: white), water molecules and ions are not shown for clarity.

are all insoluble, and solubility decreases in going from **3** to **6**. This is surprising at some level, as the behavior at the onset of globular structure—predicted here to be at generation 5—might be expected to affect this trend.

Computation offers further insight into the role of these linkers in solvation. Molecular models of **2–4** were immersed in a periodic simulation box containing explicit water molecules and 150 mM NaCl and were investigated by means of molecular dynamics (MD) simulations. Equilibrated configurations of **2** and **4** in solution were obtained within 250 ns. Dendrimer **3** required a longer equilibration time, and the MD simulation in this case lasted 400 ns (see ESI†). The equilibrated configurations of **2**, **3** and **4** shown in Fig. 1 reflect the color scheme adopted throughout the manuscript with cyan representing trismethylene bispiperidine and orange representing 4,7,10-trioxotridecane-1,14-diamine.

Computation reveals differences that are consistent with the solubility trend observed. In all cases, the hydrophobic core is sufficiently rigid that complete hydrophobic collapse is precluded. Instead, **2** displays an extended hydrophobic surface that leads us to hypothesize a role in promoting precipitation. For **3** and **4**, the 4,7,10-trioxotridecane-1,14-diamine groups collapse to partially shield hydrophobic domains. This behavior is consistent with previous simulations⁹ and typical of the behaviour of PEG chains in water.¹⁰ The shapes of **3** and **4** differ: **3** appears more spherical/globular while **4** adopts an elongated oval shape. Table 1 summarizes computed parameters for **2**, **3** and **4**. At the equilibrium, **3** has smaller radius of gyration (R_g) than **2**. We hypothesize the difference derives from the flexibility differences between 4,7,10-trioxotridecane-1,14-diamine and trismethylene bispiperidine. This difference is reflected in the solvent accessible surface area (SASA): **2** has a

Table 1 Structural features of dendrimers **2–4** obtained from the equilibrated phase MD simulations

Dendrimer	MW (Da)	R_g (Å)	SASA (Å ²)	Density ^a (Da Å ⁻³)	H (kcal mol ⁻¹)
2	10 487	19.2	10 193	0.35	−11 254 ± 26
3	10 646	17.5	9409	0.47	−12 812 ± 35
4	14 940	21.2	13 116	0.37	−15 914 ± 39

^a Density parameter is calculated by dividing the dendrimer MW for the volume of a sphere with radius R_g .

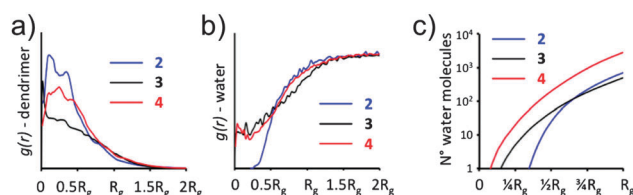


Fig. 2 Radial distribution functions, $g(r)$, obtained from the equilibrated phase MD simulations. The $g(r)$ curves are indicative of the probability to find atoms of the dendrimers (a) and water molecules (b) at given distance from the dendrimer's center of mass (CM). (c) Number of water molecules.

slightly larger SASA than 3 (10 193 Å² versus 9049 Å², respectively). The SASA of 4 is greater as would be inferred from the depiction, 13 116 Å² and consistent with the larger R_g of 21.2 Å.

The enthalpy (H) values of the three dendrimers were calculated from the equilibrated phase MD simulations. A relative comparison of H shows that 2 is least stable (relatively) in water. Dendrimers 3 and 4 have more favorable (negative) H values indicating higher relative stability. By comparing calculated H values, we can quantify differences in stability (ΔH) of the dendrimers with respect to 2. With similar numbers of atoms, 2 and 3 differ in enthalpy with $\Delta H = -1558 \pm 61$ kcal mol⁻¹, indicating that the substitution of trimethylene bispiperidine with 4,7,10-trioxotridecane-1,14-diamine has positive effect on the stability of 3 in water. Consistently, the ΔH of 4 is even more favourable difference with $\Delta H = -4659 \pm 66$ kcal mol⁻¹.

Radial distribution functions $g(r)$ were calculated from the equilibrated phase MD trajectories to probe hydration of 2, 3 and 4 (Fig. 2). The $g(r)$ curves represent the relative probability to find dendrimer atoms (Fig. 2a) or water molecules (Fig. 2b) at a given distance from the dendrimer's center of mass (CM). Higher $g(r)$ peaks correspond to a high density of atoms and/or restricted migration, while low and broad peaks are indicative of higher molecular flexibility. In all cases, distances from CM are expressed in R_g units to allow comparison between different size dendrimers.

Fig. 2 reveals poorer solvation for 2 compared with 3 and 4. In fact, the $g(r)$ curves show the dense core of 2 (Fig. 2a, blue) reduces the probability of hydration to 0 at distances lower than $\approx 1/2R_g$ (Fig. 2b). On the other hand, the same results demonstrate that 3 and 4 have a higher levels of core hydration. Fig. 2c plots the number of water molecules present in the interior of the dendrimers. This result corroborates experimental observations of solubility with the solvent penetration of $4 > 3 > 2$.

Conclusions

Solubility remains one of the most significant challenges in the synthesis of triazine dendrimers and limits the generation that these architectures can reach. Here, we show that solubility is impacted substantially by the choice of linking and surface groups across a range of dendrimer sizes. Piperazine, though inexpensive and highly reactive, does little to convey solubility of architectures in water when appearing on either the

periphery or interior. This sensitivity to the source of cation is somewhat curious. Flexibility, as offered by trimethylene bispiperidine, improves solubility only slightly while maintaining high reactivity. The emergence of 4,7,10-trioxotridecane-1,14-diamine as a useful building block for dendrimers comes as a surprise: primary amines sacrifice reactivity. Moreover, the introduction of hydrogen bond donors should seemingly promote aggregation, especially in common organic solvents. However, using 11, the resulting protected dendrimers are soluble in polar organic solvents including chloroform, dichloromethane, DMSO, ethyl acetate, tetrahydrofuran, dioxane, and methanol. These targets are not soluble in ether (which facilitates purification given the solubility of 11 in ether) or hexanes. The deprotected dendrimers containing 11 are soluble in the same subset of organic solvents with the exception of ethyl acetate.

The results obtained here are consistent with our recent success in reaching virus-sized dendrimers of generation 13.¹¹ There, 4,7,10-trioxotridecane-1,14-diamine was used exclusively as the linking diamine. Surmountable challenges to solubility in water appeared at generation 9, persisted through 11, and were deemed limiting at 13. When 4,7,10-trioxotridecane-1,14-diamine and piperazine linkers are alternated at each generation, dendrimers up to generation 9 were obtained.¹² Generation 11 materials were insoluble in both the protected form (in common organic solvents) and deprotected (in water).

The criteria for diamine choice continue to be refined. While these studies suggest that solubility of an advanced dendrimer might be rescued using macromonomers such as 11, there appears to be additional room for improvement. Diamines that confer the reactivity of constrained secondary amines like piperazine and trimethylene bispiperidine and retain the advantageous solubility properties of 4,7,10-trioxotridecane-1,14-diamine could prove optimal in our pursuit of the rapid synthesis of large triazine dendrimers. Further experiment and computation will be required to meet these challenges.

Experimental

Molecular dynamics (MD) simulation

The simulation work was conducted using the AMBER 12 software.¹³ The molecular models for 2, 3, and 4 dendrimers were created and parameterized according to a validated procedure used previously for similar derivatives.^{9,14} In particular, 2, 3, and 4 dendrimers were parameterized with the "general AMBER force field (GAFF)" (*gaff.dat*).¹⁵ The *parm99* all-atom force field (*leaprc.ff99*)¹⁶ was used to parameterize all the other standard residues present in the simulated molecular systems.

The models of the three dendrimers were placed in a periodic box containing explicit TIP3P water molecules¹⁷ and the necessary number of ions to neutralize the systems and reproduce the experimental ionic strength of 150 mM [NaCl]. Each system underwent initial minimization, and further heating through 50 ps of NVT MD simulation to reach the temperature of 300 K. During this second step the solute was maintained as fixed and the solvent was relaxed. Following to this phase, all systems

were equilibrated by running *NPT* MD simulations at the temperature of 300 K and 1 atm of pressure under periodic boundary conditions using a time step of 2 femtoseconds. The Langevin thermostat, and a 8 Å cutoff were used for all equilibration runs. The particle mesh Ewald¹⁸ (PME) approach was adopted to treat the long-range electrostatic effects, and all bonds involving hydrogen atoms were treated by means of the SHAKE algorithm.¹⁹ All MD simulations were carried out using the *pmemd.cuda* module of AMBER 12 working on GTX580 GPU cards. The root mean square deviation (RMSD) and radius of gyration (R_g) data were extracted from the MD trajectories with the *ptraj* module of AMBER 12 and were used to assess the equilibration of each dendrimer (see ESI†). In this respect, while a simulation time of 250 ns was enough for 2 and 4 to reach the equilibrium with good stability, having a sufficiently long equilibrated phase to allow for satisfactory analysis of the MD trajectory (the last 100 ns of MD simulations), a longer simulation time was necessary for 3 (400 ns).

The enthalpy (H) values for 2–4 dendrimers were calculated directly from the equilibrated phase MD trajectories according to the MM-PBSA approach.²⁰ H is the sum of the total gas-phase *in vacuo* non-bond energy (ΔE_{gas}) of the dendrimers and of a solvation term ($\Delta G_{\text{solv}} = \Delta G_{\text{PB}} + \Delta G_{\text{NP}}$).²¹ The polar component of ΔG_{PB} was calculated according to the Poisson–Boltzmann²² (PB) approach with a numerical solver implemented in the *pbsa* program of AMBER 12.²³ The non-polar contribution to the solvation energy was calculated as $\Delta G_{\text{NP}} = \gamma(\text{SASA}) + \beta$, in which $\gamma = 0.00542 \text{ kcal } \text{\AA}^{-2}$, $\beta = 0.92 \text{ kcal mol}^{-1}$, and the solvent-accessible surface area (SASA) was estimated with the MSMS program.²⁴

General procedure for deprotection

Compound **1-Boc** (0.417 g, 0.125 mmol) is dissolved in concentrated HCl (3 mL) and methanol (3 mL) and stirred for 15 h at room temperature. After evaporating the reaction mixture under vacuum, the residue is dissolved in dichloromethane, washed with 5 M NaOH (aq), and passed through a phase separator (Whatman). Evaporation of the organic phase yields **1** (0.316 g, quantitative).

General procedure for addition of macromonomer

A solution of **1** (0.160 g, 0.063 mmol), **9** (1.44 g, 1.01 mmol), and diisopropylethylamine (0.19 mL, 1.0 mmol) in 0.6 mL of THF is stirred at 75 °C for 6 days in a pressure relief reaction vial. After evaporation of the reaction mixture, the residue was dissolved in dichloromethane and washed with brine. The organic layer was passed through a phase separator (Whatman), and evaporated under vacuum. The solid was purified by silica gel chromatography (100% dichloromethane to 9:1 dichloromethane:methanol). Compound **12** (0.576 g, 67%)—the protected precursor of **5**—was recovered as a white solid.

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Accelerated synthesis of large generation triazine dendrimers using microwave assisted reactions: a 24 hour challenge†

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The expedited synthesis of odd generation triazine dendrimers up to generation 9 can be executed in high yields using microwave irradiation. The efforts commence from commercially-available and inexpensive materials. Execution is facilitated by automated chromatography.

Introduction

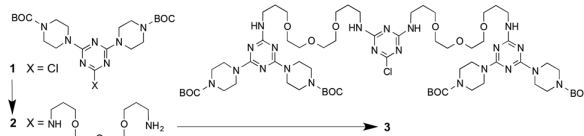
Unlike linear polymers that are available in one step through polymerization of monomer(s), the synthesis of dendrimers relies on multiple steps. While burdensome in terms of both scale and time, strategies to reduce these tolls continue to be advanced.¹ In 2010, Hawker and Malkoch reported the synthesis of a generation 6 dendrimer in less than a day exploiting thiol-ene reactivity and click chemistry.² Two different, orthogonal monomers were employed. One monomer presented one thiol and two azides. The other presented two alkenes and an alkyne. The iterative synthesis produced highly monodisperse materials at low generations ($PDI \leq 1.03$) and the onset of low polydispersity at generations 5 and 6. The only drawback to the strategy rested in the preparation of the monomers which entailed six overnight reactions and four chromatographic purifications. More recently, Malkoch *et al.* achieved a generation 6 dendrimer derived from bismethylolpropionic acid in less than a day (including the synthesis of starting materials) using the reactivity of carbonyldiimidazole and CsF as a catalyst.³

These efforts punctuate a long standing challenge to the community—the rapid and facile synthesis of dendrimers. To this end, many different approaches have been pursued.⁴ In most of the cases, hypermonomers are employed that exploit the simplicity of Michael additions,⁵ acid-amine conjugations,⁶ thiol-ene photoadditions⁷ and click chemistry.⁸ The “onion peel” dendrimers described by Roy *et al.* provide a noteworthy example.⁹ Our own efforts in accelerating the syn-

thesis of triazine dendrimers using microwave irradiation have inspired us to take on this “24 hour challenge” to synthesis. We have shown that microwave irradiation substantially decrease times of reaction in low generation dendrimers synthesized by convergent route.¹⁰ In light of this success and motivated by the long standing interest of performing an easy and fast synthesis, we decide to extend this chemistry to large generation triazine dendrimers using a divergent approach. We have shown that triazine dendrimers may have potential applications in many areas based on the ability to create versatile structures including areas in gene and drug delivery as well as materials science.¹¹

Results and discussion

The synthesis strategy employed here to reach generation 9 dendrimers utilizes a macromonomer, **3**, that affords two generations per iterative reaction cycle (Scheme 1).¹² This macromonomer comprises hydrophilic linkers based on 4,7,10-trioxa-1,13-tridecanediamine and BOC-piperazine groups. Previous studies have established that piperazine and other constrained secondary amines provide the necessary reactivity for substitution of a monochlorotriazine.¹³ All three building blocks employed; BOC-piperazine, cyanuric chloride, and 4,7,10-trioxa-1,13-tridecanediamine are commercially available and used as received. Cost analysis based on yields and solvent



Scheme 1 Synthesis of macromonomer **3** from **1** and **2**. See text for details.

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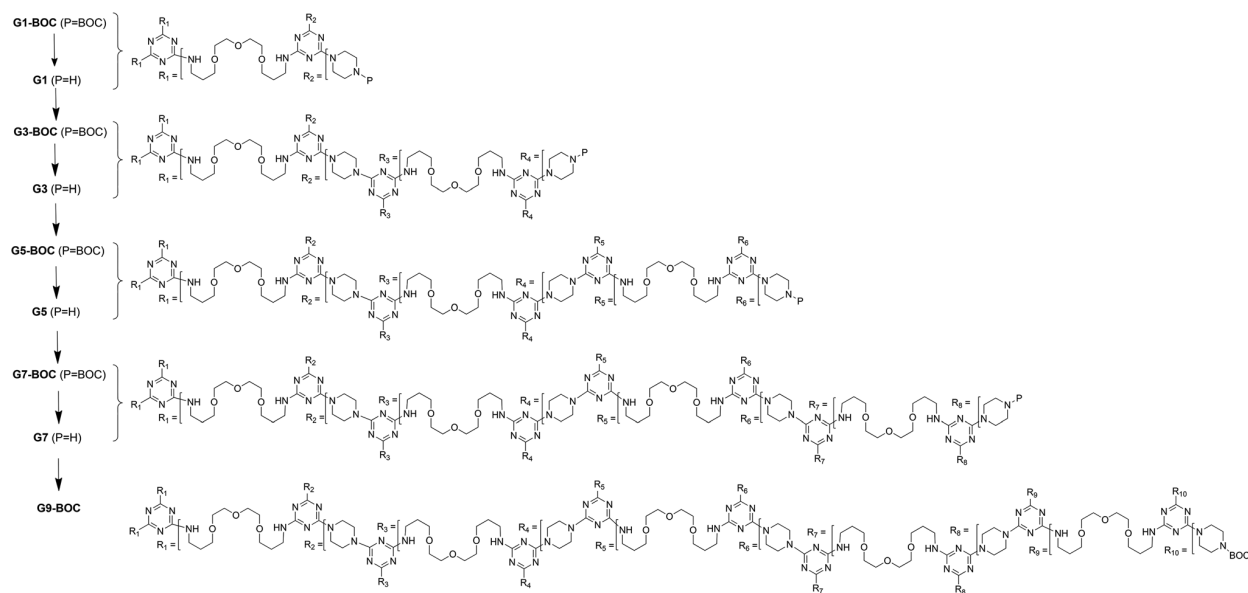


Chart 1 Dendrimers prepared in this study.

consumption for both synthesis and purification lead to a cost of \$280 per g of generation 9 dendrimer at the modest scales employed here. Macromonomer **3** is readily prepared in three steps in 50 minutes of total reaction time (Scheme 1). Specifically, cyanuric chloride was disubstituted with BOC-piperazine in tetrahydrofuran (THF) using diisopropylethylamine (DIPEA) as a base with an irradiation time of 10 minutes at 60 °C. The product, **1**, is recovered by precipitation. Next, monochlorotriazine **1** is reacted with an excess of 4,7,10-trioxa-1,13-tridecanediamine in dioxane at 95 °C for 30 minutes using cesium carbonate as a base. Following chromatography, amine **2**, was reacted with cyanuric chloride in THF at 60 °C for 10 minutes with DIPEA as a base to yield **3**. Macromonomer **3** was purified by chromatography. The overall yield for this three-step procedure is 55%.

Chart 1 shows the structures of the dendrimers prepared in this study. These materials are named by the generation number “x” as **Gx**. Intermediates carrying BOC protecting groups are identified as **Gx-BOC**. The synthesis is divergent and rests on the availability of large amounts of macromonomer **3**. To prepare the generation one dendrimer, **G1**, macromonomer **3** is reacted with additional **2**. The primary amine of **2** reacts much more sluggishly with monochlorotriazines than piperazine groups. Accordingly, in presence of an excess of **2** in dioxane with cesium carbonate as a base, the reaction requires 95 °C for 2.5 hours. The result, **G1-BOC** is obtained at 60% yield after chromatography.

Deprotection to yield **G1** in dioxane/HCl requires only 6 minutes at 60 °C in the microwave. The product is obtained quantitatively and used without further purification.

The remaining steps of the synthesis follow this iterative process of addition of **3** and acid-catalyzed deprotection.

Compd	Rxn Time	Yield (%)	Cume (%)	Iso.	Isolation Time	Ends	MW (Da)
1	10 min	82	82	Prec	1 hr	NA	484
2	30 min	70	57	Chrm	2 hr	NA	667
3	10 min	95	55	Chrm	1 hr	NA	1446
G1-BOC	2.5 h	60	33	Chrm	1 hr	6	2077
G1	6 min	Quant.	33	Extr	30 min	6	1477
G3-BOC	4 h	82	27	Chrm.	1 hr	24	9936
G3	6 min	Quant.	27	Extr	30 min	24	7535
G5-BOC	4 h	75	20	Prec	1 hr	96	41K
G5	6 min	Quant.	20	Extr	30 min	96	32K
G7-BOC	6 h	85	17	Prec	1 hr	384	167K
G7	6 min	90	15	Extr	30 min	384	128K
G9-BOC	6 h	80	12	Prec	1 hr	1536	670K

Chart 2 Targets, reaction times, yields for individual reactions and the cumulative process (cume) over the entire sequence, isolation procedure (precipitation, chromatography, or extraction), isolation time and theoretical characteristics of the products.

All addition reactions are executed at 95 °C under microwave irradiation. All deprotection reactions are executed similarly, but at 60 °C. Beyond **G1**, the solvent system used for addition of **3** is dioxane : methanol : water at 2 : 1 : 0.1. Deprotections are carried out in 2 : 1 dioxane : conc. HCl. Chart 2 summarizes the individual yields, cumulative yields, and other attributes of the target molecules. The time for addition of **3** increases as the dendrimer generation increases. However, this increase is offset by the ease of purification: **3** and product dendrimer show markedly different solubility in ether with trace methanol.

Throughout the course of the synthesis, the intermediates and targets can be characterized by ¹H and ¹³C NMR spectroscopy and mass spectrometry. ¹H NMR spectra show characteristic loss of BOC groups on deprotection, and appearance of substituted piperazine groups at δ 2.81–2.87. Mass spectrometry shows isotopic resolution and lines for materials

up to **G5-BOC**. Defects for **G5** and **G5-BOC** can be seen by ESI-MS. Larger dendrimers show a broad peak that can be attributed to incomplete reaction. Using GPC analysis, PDI values were possible to calculate for **G1-BOC**, **G3-BOC**, **G5-BOC**, and **G7-BOC** (1.05, 1.09, 1.11 and 1.18). When **G5** dendrimer was resubjected to reaction conditions with additional **3**, the PDI value did not decrease, remaining 1.11. No PDI could be obtained for **G9-BOC**.

While reaction times for synthesis of dendrimers up to **G9** might suggest that effort can be executed in under a day, purification does increase the burden. To simplify purification we use an automated flash chromatography unit that allows a total purification time of approximately 5 h for gram scale reactions for the four steps indicated. Precipitations can be performed repeatedly to incrementally increase yields as well.

Experimental

Compound 1

1-Boc-piperazine (11.14 g, 60 mmol) was added to a solution of cyanuric chloride (5.02 g, 27 mmol) in THF (200 mL). Afterwards DIPEA (19 mL, 0.109 mol) was added dropwise. The solution was stirred for 2 minutes in order to allow reagents to mix. Then, the solution was separated in multiple vessels and irradiated in the microwave while stirring for 10 minutes at 60 °C using dynamic mode. The crude product was purified by precipitations hexanes/EtOAc to give **1** (10.83 g, 82%) as a white solid. ^1H NMR (400 MHz, CDCl_3) δ 3.74 (br, 8H, $\text{NCH}_2\text{CH}_2\text{NBoc}$), 3.44 (br, 8H, $\text{NCH}_2\text{CH}_2\text{NBoc}$), 1.45 (s, 18H, $\text{C}(\text{CH}_3)_3$); ^{13}C NMR (100 MHz, CDCl_3) δ 169.6, 164.4 (C_3N_3), 156.5 (CO), 80.1 ($\text{C}(\text{CH}_3)_3$), 43.2 ($\text{NCH}_2\text{CH}_2\text{N}$), 28.3 ($\text{C}(\text{CH}_3)_3$); MS (ESI-TOF) calcd for $\text{C}_{21}\text{H}_{34}\text{ClN}_7\text{O}_4$ 483.2361, found 484.3702 ($\text{M} + \text{H}$) $^+$.

Compound 2

A solution of **1** (3 g, 6 mmol) with 4,7,10-trioxa-1,13-tridecane-diamine (13.65 mL, 62 mmol) and Cs_2CO_3 (4 g, 12 mmol) in 40 mL of 1,4 dioxane was stirred for 2 minutes. Then, the solution was separated in multiple vessels and irradiated in the microwave while stirring for 30 minutes at 95 °C and then evaporated under vacuum. The residue was dissolved in dichloromethane, washed with brine solution and dried over MgSO_4 , filtered, and evaporated under vacuum. The solvent system (in column volumes) used was the following: 5CV (100% DCM), 5CV (95 : 5 = DCM : MeOH), 5CV (90 : 10 = DCM : MeOH), 5CV (85 : 15 = DCM : MeOH), 5CV (80 : 20 = DCM : MeOH) to give **2** (3.26 g, 79%) as a white solid. ^1H NMR (400 MHz, CDCl_3) δ 3.73 (br, 8H, $\text{NCH}_2\text{CH}_2\text{NBoc}$), 3.67–3.57 (m, 12H, $\text{CH}_2\text{OCH}_2\text{CH}_2\text{OCH}_2\text{CH}_2\text{OCH}_2$), 3.44 (br, 10H, $\text{NHCH}_2\text{CH}_2\text{CH}_2\text{O}$, $\text{NCH}_2\text{CH}_2\text{NBoc}$), 2.81 (t, 2H, $\text{OCH}_2\text{CH}_2\text{CH}_2\text{NH}_2$), 1.84 (m, 2H, $\text{OCH}_2\text{CH}_2\text{CH}_2\text{NH}$), 1.76 (m, 2H, $\text{OCH}_2\text{CH}_2\text{CH}_2\text{NH}_2$), 1.47 (s, 18H, $\text{C}(\text{CH}_3)_3$); ^{13}C NMR (100 MHz, CDCl_3) δ 166.3, 165.2 (C_3N_3), 154.8 (CO), 79.8 ($\text{C}(\text{CH}_3)_3$), 70.5 ($\text{OCH}_2\text{CH}_2\text{O}$), 70.2 ($\text{OCH}_2\text{CH}_2\text{O}$), 70.1 ($\text{OCH}_2\text{CH}_2\text{O}$), 69.5 (two lines, $\text{NHCH}_2\text{CH}_2\text{CH}_2\text{O}$, $\text{OCH}_2\text{CH}_2\text{CH}_2\text{NH}_2$), 43.8 (piperazine),

39.4 ($\text{NHCH}_2\text{CH}_2\text{CH}_2\text{O}$), 38.2 ($\text{OCH}_2\text{CH}_2\text{CH}_2\text{NH}_2$), 32.6 ($\text{OCH}_2\text{CH}_2\text{CH}_2\text{NH}_2$), 29.6 ($\text{NHCH}_2\text{CH}_2\text{CH}_2\text{O}$), 28.6 ($\text{C}(\text{CH}_3)_3$); MS (ESI-TOF) calcd for $\text{C}_{31}\text{H}_{57}\text{N}_9\text{O}_7$ 667.4381, found 668.5915 ($\text{M} + \text{H}$) $^+$.

Compound 3 (macromonomer)

Compound **2** (3.26 g, 4.8 mmol) was added to a solution of cyanuric chloride (0.411 g, 2.2 mmol) in THF (20 mL). Afterwards DIPEA (3.2 mL, 18 mmol) was added dropwise, and the solution was sonicated for 2 minutes in order to allow reagents to mix. Then, the solution was irradiated in the microwave while stirring for 10 minutes at 60 °C using dynamic mode. The solvent system (in column volumes) used was the following: 5 CV (100% DCM), 5 CV (95 : 5 = DCM : MeOH), 5CV (90 : 10 = DCM : MeOH), 5CV (85 : 15 = DCM : MeOH), 5 CV (80 : 20 = DCM : MeOH) to give **3** (3.07 g, 95%) as a white solid. ^1H NMR (400 MHz, CDCl_3) δ 3.73 (br, 16H, $\text{NCH}_2\text{CH}_2\text{NBoc}$), 3.69–3.57 (m, 24H, $\text{CH}_2\text{OCH}_2\text{CH}_2\text{OCH}_2\text{CH}_2\text{OCH}_2$), 3.44 (br, 24H, $\text{C}_3\text{N}_3\text{-NHCH}_2\text{CH}_2\text{CH}_2\text{O}$, $\text{NCH}_2\text{CH}_2\text{NBoc}$), 1.86 (m, 8H, $\text{OCH}_2\text{CH}_2\text{CH}_2\text{NH}$), 1.48 (s, 36H, $\text{C}(\text{CH}_3)_3$); ^{13}C NMR (100 MHz, CDCl_3) δ 166.3 (C_3N_3), 165.7 (C_3N_3), 165.2 (C_3N_3), 154.8 (CO), 79.8 ($\text{C}(\text{CH}_3)_3$), 70.6 ($\text{OCH}_2\text{CH}_2\text{O}$), 70.3 ($\text{OCH}_2\text{CH}_2\text{O}$), 69.4 ($\text{NHCH}_2\text{CH}_2\text{CH}_2\text{O}$), 42.9 (piperazine), 38.9 ($\text{NHCH}_2\text{CH}_2\text{CH}_2\text{O}$), 38.3 ($\text{NHCH}_2\text{CH}_2\text{CH}_2\text{O}$), 29.6 ($\text{NHCH}_2\text{CH}_2\text{CH}_2\text{O}$), 28.8 ($\text{NHCH}_2\text{CH}_2\text{CH}_2\text{O}$), 28.4 ($\text{C}(\text{CH}_3)_3$); MS (ESI-TOF) calcd for $\text{C}_{65}\text{H}_{112}\text{ClN}_{21}\text{O}_{14}$ 1445.8386, found 1447.1735 ($\text{M} + \text{H}$) $^+$.

Compound 4 (G1-Boc)

A solution of **3** (0.743 g, 0.5 mmol) with **2** (1.042 g, 2 mmol) and Cs_2CO_3 (1.066 g, 3 mmol) in 5 mL of 1,4 dioxane and 0.5 mL MeOH was stirred for 2 minutes. Then, the solution was irradiated in the microwave while stirring for 2 hours 30 minutes at 95 °C using dynamic mode and then evaporated under vacuum. The residue was dissolved in dichloromethane, washed with brine solution and dried over MgSO_4 , filtered, and evaporated under vacuum. The crude was purified by automated chromatography. The solvent system (in column volumes) used was the following: 20CV (100% DCM), 5CV (90 : 10 = DCM : MeOH) to give **4** (0.709 g, 66%) as a white solid. ^1H NMR (400 MHz, CDCl_3) δ 3.71 (br, 24H, $\text{NCH}_2\text{CH}_2\text{NBoc}$), 3.64–3.52 (m, 36H, $\text{CH}_2\text{OCH}_2\text{CH}_2\text{OCH}_2\text{CH}_2\text{OCH}_2$), 3.43 (br, 36H, $\text{C}_3\text{N}_3\text{-NHCH}_2\text{CH}_2\text{CH}_2\text{O}$, $\text{BocNCH}_2\text{CH}_2\text{N}$), 1.83 (m, 12H, $\text{OCH}_2\text{CH}_2\text{CH}_2\text{NH}$), 1.46 (s, 54H, $\text{C}(\text{CH}_3)_3$); ^{13}C NMR (100 MHz, CDCl_3) δ 166.3, 165.26 (C_3N_3), 154.8 (CO), 79.8 ($\text{C}(\text{CH}_3)_3$), 70.6 ($\text{OCH}_2\text{CH}_2\text{O}$), 70.2 (two lines, $\text{OCH}_2\text{CH}_2\text{O}$), 69.2 (two lines, $\text{NHCH}_2\text{CH}_2\text{CH}_2\text{O}$), 42.9 (piperazine), 38.2 ($\text{NHCH}_2\text{CH}_2\text{CH}_2\text{O}$), 38.1 ($\text{NHCH}_2\text{CH}_2\text{CH}_2\text{O}$), 29.6 ($\text{NHCH}_2\text{CH}_2\text{CH}_2\text{O}$), 28.4 ($\text{C}(\text{CH}_3)_3$); MS (ESI-TOF) calcd for $\text{C}_{96}\text{H}_{168}\text{N}_{30}\text{O}_{21}$ 2077.3000, found 2079.6681 ($\text{M} + \text{H}$) $^+$.

Compound 5 (G1 deprotected)

A solution of **4** (0.800 g, 0.385 mmol) in concentrated HCl (3 mL) and 1,4 dioxane (6 mL) was stirred for 1 min at room temperature and then was irradiated in the microwave while stirring for two periods of 3 minutes at 60 °C using dynamic mode and then evaporated with air. The residue was dissolved

in dichloromethane, washed with 5 M NaOH (aq.), dried over MgSO_4 , filtered, and evaporated under vacuum to give **5** (0.571 g, quantitative) as a white solid. ^1H NMR (400 MHz, CDCl_3) δ 3.69 (br, 24H, $\text{NCH}_2\text{CH}_2\text{NH}$), 3.64–3.51 (m, 36H, $\text{CH}_2\text{OCH}_2\text{CH}_2\text{OCH}_2\text{CH}_2\text{OCH}_2$), 3.40 (br, 12H, $\text{C}_3\text{N}_3\text{-NHCH}_2\text{-CH}_2\text{CH}_2\text{O}$), 2.83 (br, 24H, $\text{HNCH}_2\text{CH}_2\text{N}$), 1.82 (m, 12H, $\text{OCH}_2\text{CH}_2\text{CH}_2\text{NH}$); ^{13}C NMR (100 MHz, CDCl_3) δ 166.3, 165.2 (C_3N_3), 70.6 ($\text{OCH}_2\text{CH}_2\text{O}$), 70.2 (two lines, $\text{OCH}_2\text{CH}_2\text{O}$), 69.3 ($\text{NHCH}_2\text{CH}_2\text{CH}_2\text{O}$), 69.2 ($\text{NHCH}_2\text{CH}_2\text{CH}_2\text{O}$), 46.0 ($\text{NCH}_2\text{CH}_2\text{NH}$), 44.2 ($\text{NCH}_2\text{CH}_2\text{NH}$), 38.2 ($\text{NHCH}_2\text{CH}_2\text{CH}_2\text{O}$), 38.1 ($\text{NHCH}_2\text{CH}_2\text{CH}_2\text{O}$), 29.6 ($\text{NHCH}_2\text{CH}_2\text{CH}_2\text{O}$); MS (ESI-TOF) calcd for $\text{C}_{66}\text{H}_{120}\text{N}_{30}\text{O}_9$ 1476.9855, found 1478.2639 ($\text{M} + \text{H}$) $^+$.

Compound 6 (G3-Boc)

A solution of **3** (2.35 g, 1.624 mmol) with **5** (0.200 g, 0.135 mmol) and DIPEA (0.42 mL, 2.44 mmol) in 4 mL of 1,4 dioxane and 0.5 mL MeOH was stirred for 2 minutes. Then, the solution was irradiated in the microwave while stirring for 4 hours at 95 °C using dynamic mode and then evaporated under vacuum. The residue was dissolved in dichloromethane, washed with brine solution and dried over MgSO_4 , filtered, and evaporated under vacuum. The crude was purified by automated chromatography. The solvent system (in column volumes) used was the following: 5CV (99 : 1 = EtoAc : MeOH), 5CV (98 : 2 = EtoAc : MeOH), 5CV (100% DCM) to give **6** (1.1 g, 82%) as a white solid. ^1H NMR (400 MHz, CDCl_3) δ 3.72 (br, 144H, $\text{NCH}_2\text{CH}_2\text{NBoc}$, $\text{NCH}_2\text{CH}_2\text{N}$), 3.66–3.53 (m, 180H, $\text{CH}_2\text{OCH}_2\text{CH}_2\text{OCH}_2\text{CH}_2\text{OCH}_2$), 3.45 (br, 156H, $\text{C}_3\text{N}_3\text{-NHCH}_2\text{-CH}_2\text{CH}_2\text{O}$, $\text{BocNCH}_2\text{CH}_2\text{N}$), 1.86 (m, 60H, $\text{OCH}_2\text{CH}_2\text{CH}_2\text{NH}$), 1.47 (s, 216H, $\text{C}(\text{CH}_3)_3$); ^{13}C NMR (100 MHz, CDCl_3) δ 166.3, 165.26 (C_3N_3), 154.8 (CO), 79.8 ($\text{C}(\text{CH}_3)_3$), 70.6 ($\text{OCH}_2\text{CH}_2\text{O}$), 70.2 (two lines, $\text{OCH}_2\text{CH}_2\text{O}$), 69.3 (two lines, $\text{NHCH}_2\text{CH}_2\text{-CH}_2\text{O}$), 42.9 (piperazine), 38.2 ($\text{NHCH}_2\text{CH}_2\text{CH}_2\text{O}$), 38.1 ($\text{NHCH}_2\text{CH}_2\text{CH}_2\text{O}$), 29.6 ($\text{NHCH}_2\text{CH}_2\text{CH}_2\text{O}$), 28.4 ($\text{C}(\text{CH}_3)_3$); MS (ESI-TOF) calcd for $\text{C}_{456}\text{H}_{786}\text{N}_{156}\text{O}_{93}$ 9936.16, found 9944.5803 ($\text{M} + \text{H}$) $^+$.

Compound 7 (G3 deprotected)

A solution of **6** (0.650 g, 65.4 μmol) in concentrated HCl (3 mL) and dioxane (6 mL) was stirred for 1 min at room temperature and then was irradiated in the microwave while stirring for two periods of 3 minutes at 60 °C using dynamic mode and then evaporated with air. The residue was dissolved in dichloromethane, washed with 5 M NaOH (aq.), dried over MgSO_4 , filtered, and evaporated under vacuum to give **7** (0.493 g, quantitative) as a white solid. ^1H NMR (400 MHz, CDCl_3) δ 3.72 (br, 144H, $\text{NCH}_2\text{CH}_2\text{NH}$, $\text{NCH}_2\text{CH}_2\text{N}$), 3.67–3.53 (m, 180H, $\text{CH}_2\text{OCH}_2\text{CH}_2\text{OCH}_2\text{CH}_2\text{OCH}_2$), 3.47 (br, 60H, $\text{C}_3\text{N}_3\text{-NHCH}_2\text{CH}_2\text{CH}_2\text{O}$), 2.87 (br, 96H, $\text{HNCH}_2\text{CH}_2\text{N}$), 1.85 (m, 60H, $\text{OCH}_2\text{CH}_2\text{CH}_2\text{NH}$); ^{13}C NMR (100 MHz, CDCl_3) δ 166.3, 165.2 (C_3N_3), 70.6 ($\text{OCH}_2\text{CH}_2\text{O}$), 70.2 ($\text{OCH}_2\text{CH}_2\text{O}$), 69.3 (two lines, $\text{NHCH}_2\text{CH}_2\text{CH}_2\text{O}$), 46.0 ($\text{NCH}_2\text{CH}_2\text{NH}$), 44.2 ($\text{NCH}_2\text{CH}_2\text{NH}$), 43.0 ($\text{NCH}_2\text{CH}_2\text{N}$), 38.1 ($\text{NHCH}_2\text{CH}_2\text{CH}_2\text{O}$), 29.6 ($\text{NHCH}_2\text{CH}_2\text{CH}_2\text{O}$); MS (ESI-TOF) calcd for $\text{C}_{336}\text{H}_{594}\text{N}_{156}\text{O}_{45}$ 7534.90, found 7541.6290 ($\text{M} + \text{H}$) $^+$.

Compound 8 (G5-Boc)

A solution of **3** (1.84 g, 1.274 mmol) with **7** (0.200 g, 26.53 μmol) and DIPEA (0.33 mL, 1.9 mmol) in 6 mL of 1,4 dioxane, 0.5 mL MeOH and 0.5 mL H_2O was stirred for 2 minutes. Then, the solution was irradiated in the microwave while stirring for 4 hours at 95 °C using dynamic mode and then evaporated under vacuum. The residue was dissolved in dichloromethane, washed with brine solution and dried over MgSO_4 , filtered, and evaporated under vacuum. The crude was purified by several washes with a solution of 98 : 2 = EtoEt : MeOH to give **8** (0.76 g, 75%) as a white solid. ^1H NMR (400 MHz, CDCl_3) δ 3.73 (br, 624H, $\text{NCH}_2\text{CH}_2\text{NBoc}$, $\text{NCH}_2\text{CH}_2\text{N}$), 3.67–3.53 (m, 756H, $\text{CH}_2\text{OCH}_2\text{CH}_2\text{OCH}_2\text{-CH}_2\text{OCH}_2$), 3.44 (br, 636H, $\text{C}_3\text{N}_3\text{-NHCH}_2\text{CH}_2\text{CH}_2\text{O}$, $\text{BocNCH}_2\text{CH}_2\text{N}$), 1.85 (m, 252H, $\text{OCH}_2\text{CH}_2\text{CH}_2\text{NH}$), 1.48 (s, 864H, $\text{C}(\text{CH}_3)_3$); ^{13}C NMR (100 MHz, CDCl_3) δ 166.3, 165.2, 165.1 (C_3N_3), 154.8 (CO), 79.8 ($\text{C}(\text{CH}_3)_3$), 70.6 ($\text{OCH}_2\text{CH}_2\text{O}$), 70.2 (two lines, $\text{OCH}_2\text{CH}_2\text{O}$), 69.3 (two lines, $\text{NHCH}_2\text{CH}_2\text{-CH}_2\text{O}$), 42.9 (piperazine), 38.2 ($\text{NHCH}_2\text{CH}_2\text{CH}_2\text{O}$), 38.1 ($\text{NHCH}_2\text{CH}_2\text{CH}_2\text{O}$), 29.6 (two lines, $\text{NHCH}_2\text{CH}_2\text{CH}_2\text{O}$), 28.4 ($\text{C}(\text{CH}_3)_3$); MS (ESI-TOF) calcd for $\text{C}_{1896}\text{H}_{3258}\text{N}_{660}\text{O}_{381}$ 41371.59, found 41404.7224 ($\text{M} + \text{H}$) $^+$.

Compound 9 (G5 deprotected)

A solution of **8** (0.400 g, 9.67 μmol) in concentrated HCl (2 mL) and dioxane (4 mL) was stirred for 1 min at room temperature and then was irradiated in the microwave while stirring for three periods of 3 minutes at 60 °C using dynamic mode and then evaporated with air. The residue was dissolved in dichloromethane, washed with 5 M NaOH (aq.), dried over MgSO_4 , filtered, and evaporated under vacuum to give **9** (0.307 g, quantitative) as a white solid. ^1H NMR (400 MHz, CDCl_3) δ 3.68 (br, 624H, $\text{NCH}_2\text{CH}_2\text{NH}$, $\text{NCH}_2\text{CH}_2\text{N}$), 3.62–3.49 (br m, 756H, $\text{CH}_2\text{OCH}_2\text{CH}_2\text{OCH}_2\text{CH}_2\text{OCH}_2$), 3.40 (br, 252H, $\text{C}_3\text{N}_3\text{-NHCH}_2\text{CH}_2\text{CH}_2\text{O}$), 2.81 (br m, 384H, $\text{HNCH}_2\text{CH}_2\text{N}$), 1.80 (m, 252H, $\text{OCH}_2\text{CH}_2\text{CH}_2\text{NH}$); ^{13}C NMR (100 MHz, CDCl_3) δ 166.3, 165.2 (C_3N_3), 70.6 ($\text{OCH}_2\text{CH}_2\text{O}$), 70.2 ($\text{OCH}_2\text{CH}_2\text{O}$), 69.3 ($\text{NHCH}_2\text{CH}_2\text{CH}_2\text{O}$), 69.2 ($\text{NHCH}_2\text{CH}_2\text{CH}_2\text{O}$), 46.0 ($\text{NCH}_2\text{CH}_2\text{NH}$), 44.2 ($\text{NCH}_2\text{CH}_2\text{NH}$), 43.0 ($\text{NCH}_2\text{CH}_2\text{N}$), 38.1 ($\text{NHCH}_2\text{CH}_2\text{CH}_2\text{O}$), 29.6 ($\text{NHCH}_2\text{CH}_2\text{CH}_2\text{O}$); MS (ESI-TOF) calcd for $\text{C}_{1416}\text{H}_{2490}\text{N}_{660}\text{O}_{189}$ 31766.55, found 31792.1374 ($\text{M} + \text{H}$) $^+$.

Compound 10 (G7-Boc)

A solution of **3** (1.75 g, 1.2 mmol) with **9** (0.200 g, 6.3 μmol) and DIPEA (0.33 mL, 1.91 mmol) in 7 mL of 1,4 dioxane, 1 mL MeOH and 0.5 mL H_2O was stirred for 2 minutes. Then, the solution was irradiated in the microwave while stirring for 6 hours at 95 °C using dynamic mode and then evaporated under vacuum. The residue was dissolved in dichloromethane, washed with brine solution and dried over MgSO_4 , filtered, and evaporated under vacuum. The crude was purified by several washes with a solution of 97 : 3 = EtoEt : MeOH to give **10** (0.850 g, 81%) as a white solid. ^1H NMR (400 MHz, CDCl_3) δ 3.73 (br, 2544H, $\text{NCH}_2\text{CH}_2\text{NBoc}$, $\text{NCH}_2\text{CH}_2\text{N}$),

3.65–3.52 (m, 3060H, $\text{CH}_2\text{OCH}_2\text{CH}_2\text{OCH}_2\text{CH}_2\text{OCH}_2$), 3.43 (br, 2556H, $\text{C}_3\text{N}_3\text{-NHCH}_2\text{CH}_2\text{CH}_2\text{O}$, $\text{BocNCH}_2\text{CH}_2\text{N}$), 1.84 (m, 1020H, $\text{OCH}_2\text{CH}_2\text{CH}_2\text{NH}$), 1.48 (s, 3456H, $\text{C}(\text{CH}_3)_3$); ^{13}C NMR (100 MHz, CDCl_3) δ 166.3, 165.2 (C_3N_3), 154.8 (CO), 79.8 ($\text{C}(\text{CH}_3)_3$), 70.6 ($\text{OCH}_2\text{CH}_2\text{O}$), 70.2 (two lines, $\text{OCH}_2\text{CH}_2\text{O}$), 69.3 ($\text{NHCH}_2\text{CH}_2\text{CH}_2\text{O}$), 69.2 ($\text{NHCH}_2\text{CH}_2\text{CH}_2\text{O}$), 42.9 (piperazine), 38.2 ($\text{NHCH}_2\text{CH}_2\text{CH}_2\text{O}$), 29.6 ($\text{NHCH}_2\text{CH}_2\text{CH}_2\text{O}$), 28.4 ($\text{C}(\text{CH}_3)_3$); MS (ESI-TOF) calcd for $\text{C}_{7656}\text{H}_{13146}\text{N}_{2676}\text{O}_{1533}$ 167113.30, not found.

Compound 11 (G7 deprotected)

A solution of **10** (0.390 g, 2.3 μmol) in concentrated HCl (2.5 mL) and dioxane (5 mL) was stirred for 1 min at room temperature and then was irradiated in the microwave while stirring for three periods of 3 minutes at 60 °C using dynamic mode and then evaporated with air. The residue was dissolved in dichloromethane, washed with 5 M NaOH (aq.), dried over MgSO_4 , filtered, and evaporated under vacuum to give **9** (0.270 g, 90%) as a white solid. ^1H NMR (400 MHz, CDCl_3) δ 3.71 (br, 2544H, $\text{NCH}_2\text{CH}_2\text{NH}$, $\text{NCH}_2\text{CH}_2\text{N}$), 3.64–3.51 (br m, 3060H, $\text{CH}_2\text{OCH}_2\text{CH}_2\text{OCH}_2\text{CH}_2\text{OCH}_2$), 3.44 (br, 1020H, $\text{C}_3\text{N}_3\text{-NHCH}_2\text{CH}_2\text{CH}_2\text{O}$), 2.84 (br, 1536H, $\text{HNCH}_2\text{CH}_2\text{N}$), 1.83 (m, 1020H, $\text{OCH}_2\text{CH}_2\text{CH}_2\text{NH}$); ^{13}C NMR (100 MHz, CDCl_3) δ 166.3, 165.2 (C_3N_3), 70.6 ($\text{OCH}_2\text{CH}_2\text{O}$), 70.2 ($\text{OCH}_2\text{CH}_2\text{O}$), 69.3 ($\text{NHCH}_2\text{CH}_2\text{CH}_2\text{O}$), 69.2 ($\text{NHCH}_2\text{CH}_2\text{CH}_2\text{O}$), 46.0 ($\text{NCH}_2\text{CH}_2\text{-NH}$), 44.2 ($\text{NCH}_2\text{CH}_2\text{NH}$), 43.0 ($\text{NCH}_2\text{CH}_2\text{N}$), 38.1 ($\text{NHCH}_2\text{CH}_2\text{CH}_2\text{O}$), 29.7 ($\text{NHCH}_2\text{CH}_2\text{CH}_2\text{O}$); MS (MALDI-TOF) calcd for $\text{C}_{5736}\text{H}_{10074}\text{N}_{2676}\text{O}_{765}$ 128693.17, not found.

Compound 12 (G9-Boc)

A solution of **3** (0.430 g, 0.30 mmol) with **11** (0.050 g, 0.39 μmol) and DIPEA (0.08 mL, 0.45 mmol) in 3 mL of 1,4 dioxane, 1 mL MeOH and 0.5 mL H_2O was stirred for 2 minutes. Then, the solution was irradiated in the microwave while stirring for 6 hours at 95 °C using dynamic mode and then evaporated under vacuum. The residue was dissolved in dichloromethane, washed with brine solution and dried over MgSO_4 , filtered, and evaporated under vacuum. The crude was purified by several washes with MeOH to give **10** (0.209 g, 80%) as a white wax. ^1H NMR (400 MHz, CDCl_3) δ 3.71 (br, 10224H, $\text{NCH}_2\text{CH}_2\text{NBoc}$, $\text{NCH}_2\text{CH}_2\text{N}$), 3.63–3.52 (br m, 12276H, $\text{CH}_2\text{OCH}_2\text{CH}_2\text{OCH}_2\text{CH}_2\text{OCH}_2$), 3.41 (br, 10236H, $\text{C}_3\text{N}_3\text{-NHCH}_2\text{CH}_2\text{CH}_2\text{O}$, $\text{BocNCH}_2\text{CH}_2\text{N}$), 1.80 (m, 4092H, $\text{OCH}_2\text{CH}_2\text{CH}_2\text{NH}$), 1.46 (s, 13824H, $\text{C}(\text{CH}_3)_3$); ^{13}C NMR (100 MHz, CDCl_3) δ 166.2, 165.2 (C_3N_3), 154.7 (CO), 79.8 ($\text{C}(\text{CH}_3)_3$), 70.6 ($\text{OCH}_2\text{CH}_2\text{O}$), 70.2 ($\text{OCH}_2\text{CH}_2\text{O}$), 69.3 ($\text{NHCH}_2\text{CH}_2\text{CH}_2\text{O}$), 42.9 (piperazine), 38.2 ($\text{NHCH}_2\text{CH}_2\text{CH}_2\text{O}$), 29.6 ($\text{NHCH}_2\text{CH}_2\text{CH}_2\text{O}$), 28.4 ($\text{C}(\text{CH}_3)_3$); MS (MALDI-TOF) calcd for $\text{C}_{30696}\text{H}_{52698}\text{N}_{10740}\text{O}_{6141}$ 670080.15, not found.

Conclusions

Advancing dendrimers to applications requires readily available materials. While low generation triazine dendrimers have been readily available for some time,¹⁴ the efficient prepa-

ration of moderate and higher generation dendrimers has been elusive until now. Triazines now join the architectures advanced by Hawker, Malkoch, and Roy as rapidly assessable at high generations.

Acknowledgements

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Triazine-Substituted and Acyl Hydrazones: Experiment and Computation Reveal a Stability Inversion at Low pH

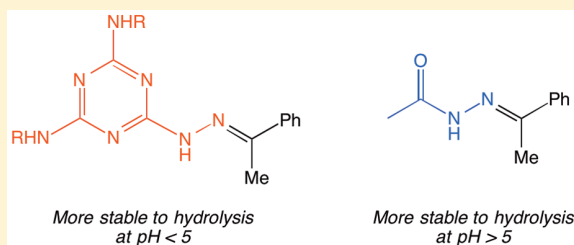
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Supporting Information

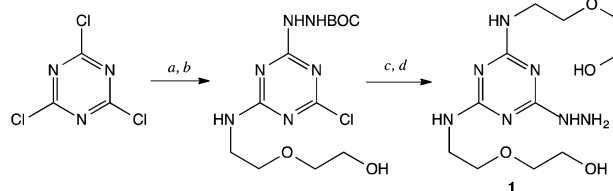
ABSTRACT: Condensation of a hydrazine-substituted *s*-triazine with an aldehyde or ketone yields an equivalent to the widely used, acid-labile acyl hydrazone. Hydrolysis of these hydrazones using a formaldehyde trap as monitored using HPLC reveals that triazine-substituted hydrazones are more labile than acetyl hydrazones at pH > 5. The reactivity trends mirror that of the corresponding acetyl hydrazones, with hydrolysis rates increasing along the series (aromatic aldehyde < aromatic ketone < aliphatic ketone). Computational and experimental studies indicate a reversal in stability around the triazine pK_a (pH ~5). Protonation of the triazine moiety retards acid-catalyzed hydrolysis of triazinyl hydrazones in comparison to acetyl hydrazone analogues. This behavior supports mechanistic interpretations suggesting that resistance to protonation of the hydrazone N1 is the critical factor in affecting the reaction rate.

KEYWORDS: triazine, hydrazine, hydrazone, pH labile, release, computation



Labile bonds in general, and hydrazones in particular, find diverse roles in chemistry ranging from materials to medical sciences. Hydrazones have been employed in the creation of dynamic combinatorial libraries¹ and as reagents and auxiliaries in organic synthesis.² Fragrant aldehydes and ketones have been incorporated into materials as pro-perfumes using hydrazone chemistry.³ Hydrazones can be bioactive in their own right,⁴ including uses as antitrypanosomals⁵ and tools for molecular biology.⁶ The lability of hydrazones in acid inspires the application of these groups as linkers in drug conjugates. Calicheamicin is conjugated to an oxidized antibody through an acyl hydrazone in Mylotarg.⁷ Conjugates of other drugs have been reported, including doxorubicin.⁸ Hydrazones have been used as the basis for switches, sensors, and other materials.⁹ While the literature is replete with examples of aliphatic, acyl, and aromatic hydrazine derivatives of triazines and the resulting hydrazones, the mechanism of hydrazone hydrolysis is unreported.¹⁰

Our longstanding interest in triazine chemistry¹¹ led us to examine whether hydrazine derivatives of triazines—so-called triazinyl hydrazines—might mirror acyl hydrazines in hydrazone formation and hydrolysis. A priori, it was unclear whether such hydrazones would be more or less stable than the acyl analogues. To start, a four-step synthesis of triazinyl hydrazine **1** (Scheme 1) was adopted. The aminoethoxyethanol substituents were included to enhance the water solubility. The moderate reactivity of BOC-NHNH₂ (BOC = *tert*-butoxycarbonyl) led to its installation on a dichlorotriazine intermediate that could be subsequently elaborated to give **1** as shown. In contrast, the reaction of cyanuric chloride with BOC-NHNH₂ led to a mixture of products, including the desired

Scheme 1. Synthesis of Triazinylhydrazine **1**^a

^aReagents and conditions: (a) Aminoethoxyethanol, THF, DIPEA, −15 to 0 °C, 1 h. (b) BOC-NHNH₂, THF, DIPEA, RT, 12 h. (c) Aminoethoxyethanol, dioxane, DIPEA, 80 °C, 12 h. (d) 4 M HCl/MeOH, 12 h.

monoaddition product, the diaddition product, and other impurities that were not identified.¹² Similarly, the elevated temperatures required for installation of BOC-NHNH₂ (or hydrazine) on a suitably derivatized monochlorotriazine led to the desired compound but also to additional impurities identified in HPLC chromatograms. Our interest in applying poly(dichlorotriazines) as intermediates in dendrimer synthesis or on other scaffolds makes this seemingly laborious synthesis generally applicable, if not desirable.¹³

We examined a suite of five aldehydes and ketones (a–e; Chart 1). We denote the hydrazone formed by the condensation of **1** and **a** as **1-a** and the corresponding acetyl

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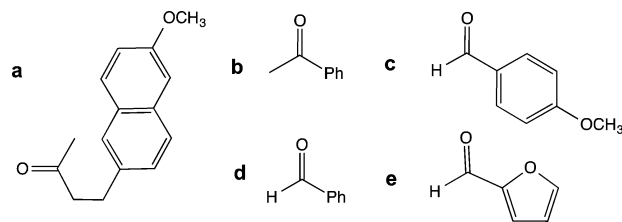
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Chart 1. Aldehydes and Ketones Used in This Study



hydrazone as **Ac-a**. The alphabetical ordering of these compounds reflects their measured stabilities toward hydrolysis (vide infra).

Condensation of **1** or acetyl hydrazine, AcNHNH_2 , to form the corresponding hydrazone was accomplished readily by mixing with a 3-fold excess of **a–e** and 0.5 equiv of acetic acid at room temperature in ethanol overnight followed by chromatographic purification. Structures were validated by ^1H and ^{13}C NMR spectroscopy as well as mass spectrometry. Each of the compounds showed a single peak in its HPLC chromatogram with a unique retention time. Hydrolysis rates were measured in a mixed solvent system to account for the varying hydrophobicity of these molecules. Briefly, 50 mM stock solutions of **1-a** through **1-e** were prepared in methanol (**Ac-a** through **Ac-e** were prepared in THF) and diluted to 10 mM with disodium phosphate/citric acid buffers to reach pH 5.2, 6.8, and 8.0. Hydrolysis was monitored in the presence of a 10-fold excess of formaldehyde as a trap¹⁴ using HPLC (see the Supporting Information). Hydrolysis was pronounced at pH 5.2. Figure 1 shows the data derived from HPLC traces. Comparatively, acyl hydrazones are more stable than their triazinyl hydrazone counterparts. The stability increases with hydrazones **a** < **b** < **c** < **d** < **e**.

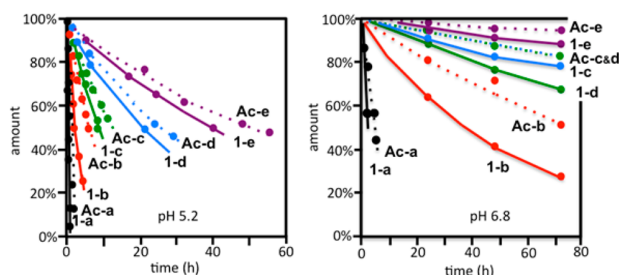


Figure 1. Hydrolysis profiles at pH 5.2 (left) and 6.8 (right).

At pH 6.8, similar trends were observed over 72 h, as shown in Figure 1, with most showing less than 20% hydrolysis over this period. At pH 8.0, negligible hydrolysis was observed over 72 h: only **1-a** and **Ac-a** showed measurable hydrolysis rates (0.0006 and 0.0001 min^{-1} , respectively). First-order reaction rate constants and half-lives are shown in Table 1.

The accepted mechanism for hydrazone hydrolysis starts with protonation of nitrogen N1 to yield **I** (Scheme 2).¹⁵ Addition of a molecule of water yields carbinolamine intermediate **II**. Proton migration to give **III** leads to C–N bond cleavage to complete the hydrolysis. Subject to historical and contemporary inquiry,^{14–17} this mechanism is consistent with catalysis observed in acid. Three different interpretations have been invoked to rationalize differences in the rates of hydrolyses of different hydrazones (and oximes and imines). These include a thermodynamic argument based on ground-state stabilities,¹⁶ resonance stabilization arguments focusing on the reduction of electrophilicity of C1,¹⁷ and stabilities derived from resistance to protonation of N1, which was recently invoked by Kalia and Raines¹⁴ to explain the relative stabilities of hydrazones and oximes.

Scheme 2. Mechanism of Hydrolysis (R = Acetyl or Triazinyl)

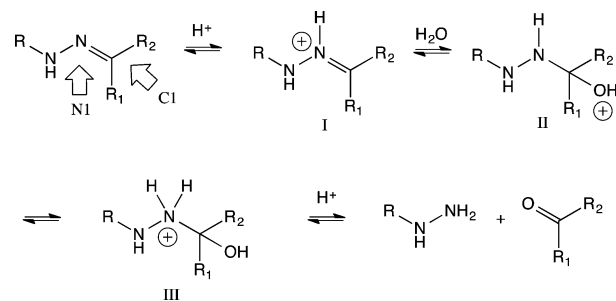


Table 1 includes density functional theory (DFT) calculations^{18–20} of proton affinities (ΔE_{prot}) of N1 of the hydrazone. These values are assigned relative to **Ac-b**. Positive values correspond to stable proton binding. The calculations used the B3LYP exchange–correlation functional,¹⁸ the 6-31+G(d,p) basis set,¹⁹ and the SMD continuum model for aqueous solvent²⁰ as implemented in the Gaussian 09 suite of programs.²¹ Other computational details and energies and geometries of all species are reported in the Supporting Information. The measured rates of hydrolysis for **Ac-a** through

Table 1. First-Order Reaction Constants and Half-Lives and DFT-Computed N1 Proton Affinities ΔE_{prot} Relative to **Ac-b**^a

compd	pH 4.0		pH 5.2		pH 6.8		ΔE_{prot} (kcal/mol) ^a
	k (10^{-3} min^{-1})	$t_{1/2}$ (min)	k (10^{-3} min^{-1})	$t_{1/2}$ (min)	k (10^{-3} min^{-1})	$t_{1/2}$ (min)	
1-a	—	—	39	18	4.7	150	4.4 (–5.0)
Ac-a	—	—	20	34	2.6	270	1.7
1-b	8.0	87	5.2	130	0.3	2310	2.3 (–4.3)
Ac-b	30	23	1.9	370	0.1	6900	0.0
1-c	1.9	360	1.3	530	0.09	—	1.0 (–4.1)
Ac-c	8.6	81	0.5	1400	0.04	—	–0.7
1-d	1.8	390	0.9	770	0.059	—	–1.3 (–4.2)
Ac-d	2.9	240	0.4	1700	0.046	—	–3.0
1-e	0.7	990	0.3	2300	0.014	—	–0.1 (–4.4)
Ac-e	3.1	224	0.2	3500	0.027	—	–1.9

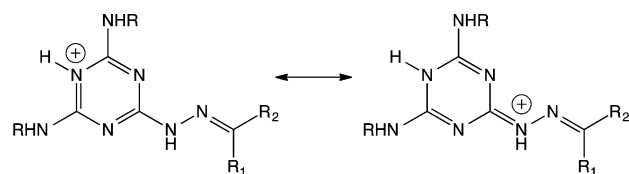
^aChanges in ΔE_{prot} upon triazine protonation are given in parentheses.

Ac-e correlate with the computed N1 proton affinities, consistent with the work of Kalia and Raines.¹⁴ For example, ketone-derived Ac-a has a shorter half-life and larger proton affinity than Ac-b, while aldehyde-derived Ac-c, Ac-d, and Ac-e have longer half-lives and smaller (more negative) proton affinities than Ac-b. Ac-c has a larger proton affinity and shorter half-life than Ac-d, consistent with an increase in the proton affinity due to resonance electron donation from the *p*-methoxy group to N1.

Table 1 also shows that the triazinyl hydrazones 1-a through 1-e all have shorter half-lives at pH 5 and larger N1 proton affinities than the corresponding acetyl hydrazones. Computation clearly supports an interpretation that resistance of N1 to protonation is the key predictor of the stability of hydrazones toward hydrolysis. Consistent with this analysis, computational models of simple imines (H_2NMe) and oximes (H_2NOMe) derived from b ($PhMeC=NMe$ and $PhMeC=NOMe$) gave a large computed N1 proton affinity (relative to Ac-b) of 14.9 kcal/mol for the rapidly hydrolyzed imine and a small proton affinity of -1.5 kcal/mol for the stable oxime.¹⁴

Triazinyl hydrazones, unlike acyl hydrazones, imines, and oximes, offer additional basic sites on the triazine. Kalia and Raines noted that protonation of N1 is disfavored across a range of pH because the pK_a of N1 is <0.7 . Accordingly, activated species will exist at very low concentrations in the pH range of interest. The pK_a of protonated triazines is ~ 5 ,²² and thus, protonated triazines must be considered at low pH. Triazine protonation should promote hydrolysis by increasing the electrophilicity of C1 through induction, but counterproductively, should increase the resistance of N1 to protonation (Scheme 3), the perceived critical event in hydrolysis.

Scheme 3. Triazine Protonation ($pK_a \approx 5$) Could Increase the Electrophilicity of C1 through Induction (Increasing the Hydrolysis Rate) or Increase the Resistance to N1 Protonation (Decreasing the Hydrolysis Rate)



Computation bears out this prediction. Table 1 shows that para protonation of the triazine in 1-a through 1-e reduces the predicted N1 proton affinity by 4–5 kcal/mol. Ortho protonation of 1-d reduces the predicted N1 proton affinity by 6–10 kcal/mol, partially as a result of steric clashes between the *o*-N–H and N1–H protons. We note that ortho protonation could conceivably accelerate hydrolysis that proceeds without N1 protonation by increasing the electrophilicity of C1. Test calculations suggested that this is a small effect. The reaction of C1 of molecule 1-d with hydroxide is made 45 kcal/mol more favorable by N1 protonation but only 11 kcal/mol more favorable by ortho protonation.

To explore this effect, we measured the rates of hydrolysis of 1-b and Ac-b at pH 4. Indeed, an inversion in stability was observed, with Ac-b hydrolyzing more rapidly than 1-b (Figure 2). Specifically, at pH 4, the rate constants (in units of 10^{-3} min^{-1}) for 1-b, Ac-b, 1-c, and Ac-c are 8.4, 2.7, 1.9, and 0.9,

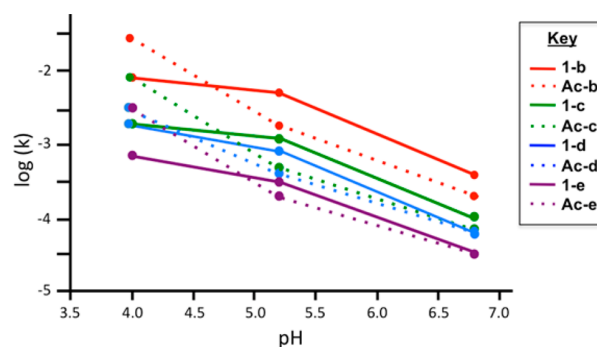


Figure 2. Hydrolysis profiles at pH 4 in the presence of a 10-fold molar excess of formaldehyde.

respectively, and the half-lives (in min) for 1-b, Ac-b, 1-c, and Ac-c are 83, 25, 364, and 81, respectively.

In summary, the data presented here support the Kalia/Raines mechanism for hydrolysis. This “resistance to protonation” hypothesis is further supported by the inversion of stability seen in triazinyl and acetyl hydrazones at low pH. This inversion in stability occurs at a pH that is biologically relevant—that associated with the cellular uptake and the endosome. Whether it can be exploited advantageously for the delivery of drugs or other agents remains to be seen.

■ ASSOCIATED CONTENT

§ Supporting Information

Synthetic details; characterization data, including spectra and kinetic studies; and a text file containing computational details and computed structures and energies. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.molpharmaceut.5b00205.

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Notes

The authors declare no competing financial interest.

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■ DEDICATION

E.E.S. dedicates this manuscript to the memory of Professor John L. Hogg, decorated and tireless educator of Texas A&M University who showed a proclivity for protons and hydrolysis reactions.²³

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Article

Functionalization of a Triazine Dendrimer Presenting Four Maleimides on the Periphery and a DOTA Group at the Core

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Abstract: A readily and rapidly accessible triazine dendrimer was manipulated in four steps with 23% overall yield to give a construct displaying four maleimide groups and DOTA. The maleimide groups of the dendrimer are sensitive to hydrolysis under basic conditions. The addition of up to four molecules of water can be observed via mass spectrometry and HPLC. The evolution in the alkene region of the ^1H -NMR—the transformation of the maleimide singlet to the appearance of two doublets—is consistent with imide hydrolysis and not the Michael addition. The hydrolysis events that proceeded over hours are sufficiently slower than the desired thiol addition reactions that occur in minutes. The addition of thiols to maleimides can be accomplished in a variety of solvents. The thiols examined derived from cysteine and include the protected amino acid, a protected dipeptide, and native oligopeptides containing either 9 or 18 amino acids. The addition reactions were monitored with HPLC and mass spectrometry in most cases. Complete substitution was observed for small molecule reactants. The model peptides containing nine or eighteen amino acids provided a mixture of products averaging between 3 and 4 substitutions/dendrimer. The functionalization of the chelate group with gadolinium was also accomplished easily.

Keywords: dendrimer; triazine; DOTA; maleimide; peptide; bioconjugate; theranostic

1. Introduction

The term “theranostics” describes molecules that offer both therapeutic and diagnostic capabilities [1–6]. Theranostic small molecules include a reporting group—commonly a chelate for MRI or PET imaging—ligated to a pharmacophore. Theranostic nanoparticles elaborate this design with materials that can offer multivalent displays of the therapeutic and/or diagnostic agent(s), as well as leverage additional properties conveyed with size, including changes in biodistribution and targeting. Theranostic nanoparticles come in many varieties including magnetic [7], silica [8–10], gold/quantum dots [11–13], graphene [14], liposomal [15,16], and polymeric [17,18]. Dendritic materials offer a compelling platform in this area [19,20] given the opportunity to exquisitely control chemistry to manipulate any number of properties displayed across multiple nanoparticle classes, so-called nanopericodicity [21–23], including size, ligand density and solubility. Versatility is a key criterion for the design of such targets. Here, we report on a tetravalent platform, **1** (Figure 1). Target **1** comprises three different domains: a reporter domain, a functional domain, and a dendritic domain. The reporter domain presents a DOTA group that can host metals for diagnostic applications such as PET or MRI. The functional domain presents four maleimides that can be readily reacted with thiols. The functional domain is linked to the reporter domain through the dendritic domain. Here, the dendritic domain is a small, generation-1 triazine dendron.

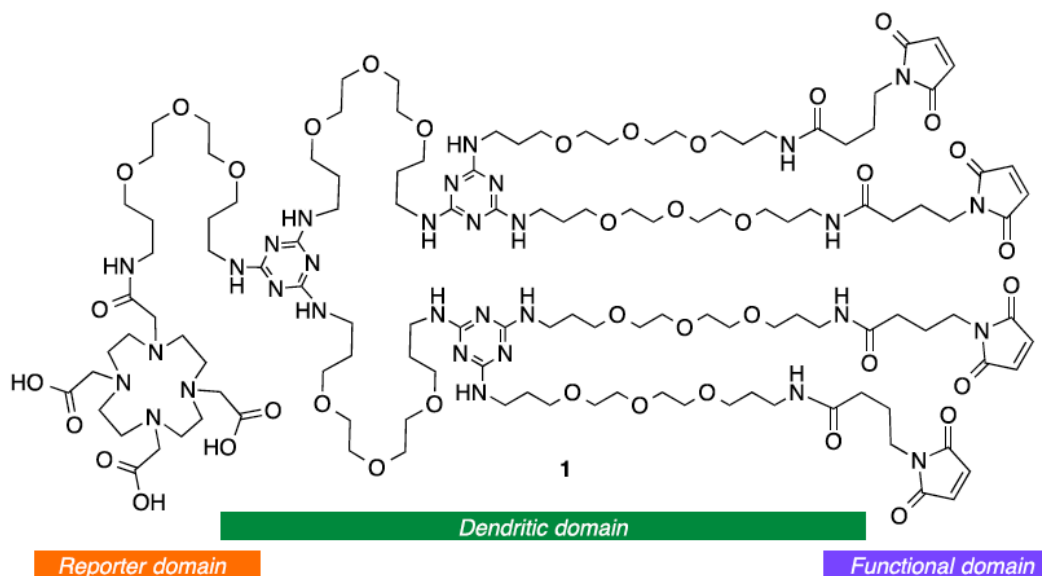


Figure 1. Target 1 with domains identified as reporter domain (orange), dendritic domain (green) and functional domain (blue).

2. Results and Discussion

2.1. Synthesis

The synthesis of 1 is shown in Scheme 1. It commences with the triazine dendron, 2, which has been previously reported [24]. Intermediate 2 was obtained in less than 24 h using microwave-assisted reactions. The installation of the DOTA-reagent, 3, was accomplished with HBTU to provide 4 in 43% yield. While mass spectrometry confirmed the structure of the product, the multiple conformations of the DOTA group led to broad signals in the ^1H -NMR spectra. The lines corresponding to the seven expected *tert*-butyl groups were well resolved, and integration matched expectation when compared with signature regions of the dendrimer.

To probe generality, and assess opportunities for potential future efforts that elaborate the dendritic portion in the presence of the reporter domain, we explored the feasibility of a two-step deprotection strategy. First, the Boc groups of 4 were selectively removed with a 1:1 mixture of 6 N HCl:MeOH to afford 5 in 79% isolated yield. Second, intermediate 5 was elaborated into a larger G3 dendrimer with 16 terminal groups (see Supplementary Materials). Then, intermediate 6 was obtained directly from 4 with a global deprotection step using a 1:1 mixture of TFA: CH_2Cl_2 . Finally, the installation of the maleimides to produce 1 proceeded with 69% yield. This four-step synthetic sequence (starting from 4) is executed with 23% overall yield. Gadolinium can be incorporated into 1 at this point (see Supplementary Materials).

2.2. Hydrolytic Stability

The hydrolytic stability of 1 in an aqueous solution is of significant concern: Commercially available maleimide crosslinking reagents are moisture sensitive, especially at $\text{pH} > 7.5$. To assess, stability, 1 was added to slightly basic water ($\text{pH} 7.5$) and monitored by mass spectrometry and HPLC. After 4 h, the addition of up to four water molecules was clearly visible by both techniques. HPLC suggests that approximately 10% of 1 remains after 4 h under these slightly basic conditions. Under neutral conditions, however, the hydrolysis is much slower: Analysis of the ^1H -NMR suggests only 25% hydrolysis, observed after 2 days.

^1H -NMR of the hydrolysis product is consistent with imide hydrolysis to a ring-opened, maleic acid amide. The alkene singlet of 1 appearing at 6.7 ppm is replaced with two doublets at 6.2 and

efficiency as determined by reaction times and product distributions. Since we predicted that **1** could serve as a building block for libraries, peptides **C** and **D** were used without extensive purification. Specifically, HPLC and MS analysis suggest that each peptide is present in greater than 60% in the crude cocktail obtained after precipitation of the cleavage product derived from a peptide synthesizer. To identify conjugates, the letter indicating the thiol and valency (when appropriate) is appended to the parent maleimide. That is, reaction of **7** and **A** produces **7-A₁** (or just **7-A**), whereas the product of three additions of **D** to **1** is identified as **1-D₃**.

A FmocCysOMe

C H₂N-CYGPPPPPG-COOH

B FmocCysTrpOMe

D H₂N-CGFQLRQPPLVSRKGEG-COOH

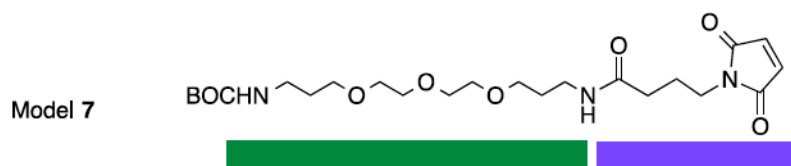


Figure 2. Thiols **A–D** in this study and model system **7** which models the dendritic domain (green) and functional domain (blue).

Compound **7** was prepared as a model to identify indicators of a successful reaction (Figure 2). The reactions of **7** with **A** (FmocCysOMe) and **B** (FmocCysTrpOMe) to yield conjugates **7-A** and **7-B**, respectively, were followed using NMR spectroscopy, mass spectrometry, and HPLC. Here, methylene chloride was used as a reaction solvent due to the limited water solubility of the protected amino acids. NMR spectroscopy provides convenient handles for assessing conjugation. The ¹H-NMR spectra showed a shift of the broad multiplet of the β-CH₂ of cysteine from ~3.0 ppm to 3.2 and 3.5 ppm with a pronounced diastereotopic splitting. Unfortunately, this region of the spectra was congested. The signals generated upon addition to the maleimide were more diagnostic. The methylene of the thioether appeared at 3.8 ppm. The adjacent methylene split into a diastereotopic pair at ~2.5 ppm (in an uncrowded region of the spectrum) and 3.1 ppm (in a more crowded region). The ¹³C-NMR was used to corroborate the addition. The appearance of diastereomers corresponding to the maleimide thioether (~39 ppm), imide methylene (~36 ppm) and β-CH₂ of the cysteine residue (~34 ppm) were diagnostic for successful conjugation of the chiral thiol. The data from mass spectrometry and HPLC were consistent with a clean reaction between **7** and **A** or **B**. Mass spectrometry of the conjugates of **7-A** and **7-B** showed the parent ion with minor ions corresponding to the loss of both the Boc and Fmoc groups. Both **7-A** and **7-B** were isolated using column chromatography and obtained with approximately 60% yields. These low yields are attributed to the loss of Fmoc group during isolation.

Oxidation of the thiol offered the potential for a competing side-reaction. The cysteine disulfide of **A** appeared at 3.25 ppm in a crowded region of the ¹H-NMR spectrum. However, HPLC analysis provided a clear indication of this compound: The disulfides, thiols and conjugation products **7-A** and **7-B** showed unique retention times (See Supplementary Materials). Oxidation to the disulfide did not appear to be responsible for low yields of isolated materials. Accordingly, no special handling procedures were adopted for latter conjugation reactions.

Advantageously, we were able to perform conjugations with **1** in a range of solvents, because **1** was readily soluble in chloroform, dichloromethane, dioxane, methanol and water. For example, reactions of **1** with hydrophobic models **A** and **B** were performed in a dioxane:dichloromethane mixture. A dioxane:water mixture was used for nonapeptide **C**. For the hydrophilic peptide **D**, the conjugation was performed in water.

Conjugates **1-A₄** and **1-B₄** were isolated using chromatography with 65% and 54% yields. Mass spectrometry, and HPLC and NMR spectroscopy all provided corroborating data. HPLC showed that the retention times of thiol **A** and the conjugation product **1-A₄** were different by approximately

1 min. However, thiol B and the product of conjugation, 1-B₄, showed similar retention times. The conjugation reaction using A and B were readily monitored by electrospray ionization-time of flight mass spectrometry, ESI-TOF MS (Figure 3a,b). Immediately upon mixing reagents, mass spectrometry showed clear evidence of conjugate formation with amino acid A reacting faster than dipeptide B.

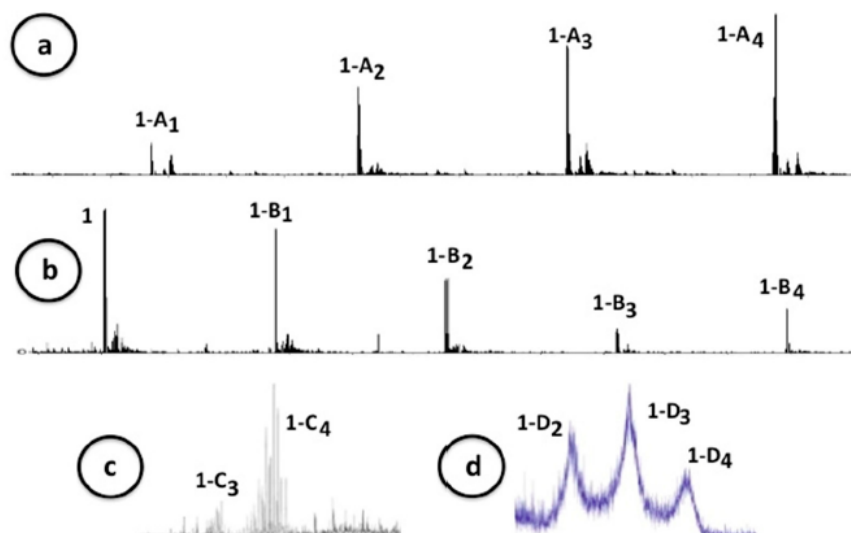


Figure 3. Conjugates 1 with A (a); B (b); C (c) and D (d). Traces (a,b) are taken upon the addition of thiol by ESI-TOF MS. Mass scales for these traces are m/z 2900–4400 (a) and m/z 2600–5200 (b); Traces (c,d) are taken of the purified product mixture. Mass scales for these traces are m/z 4500–7800 (c) and m/z 5000–13000 (d). Trace (d) was obtained by MALDI-TOF-MS.

2.4. Conjugation of Peptides

The products of conjugation of 1 with nonapeptide C were isolated in a multistep sequence. First, the reaction mixture was concentrated to an oil. The oil was then resuspended in deionized water to facilitate membrane filtration with a cellulose centrifugation filter (Ultracell YM3) with a 3 kDa molecular weight cutoff. After multiple rinses, the recovered volume was lyophilized to dryness. Using ¹H-NMR, a comparison of the integration of the aromatic signals of the tyrosine with signals derived from the methylene, which was derived from the maleimide at 2.5 ppm (dendrimer), suggests that the product distribution approached the desired 1-C₄ target, approximately ~1-C_{3.75}. Mass spectrometry confirmed a successful reaction (Figure 3c). The presence of multiple lines at lower molecular weights than the target, 1-C₄, is consistent with the use of an impure peptide.

Not surprisingly, these peaks were assignable (Figure 4). The desired product containing four copies of C is indicated with *four* green dots. Adducts of 1-C₄ with Na⁺ and K⁺ are shown with blue arrows. For lines corresponding to products with lower molecular weights, one or more of the peptides are missing amino acids. The most abundant ion displays a peptide missing a glycine residue (blue dot). Peptides missing proline (red dot) are also present. For simplicity, we indicate the minimum number of deletions for each peptide. That is, whether the final compound has one deletion in each peptide—or comprises three perfect peptides C and a peptide missing four amino acids—is unknown. The necessity of using double couplings during the solid phase synthesis of this proline-rich sequence is also reflected by the presence of a peak corresponding to a product with three copies of C and one copy of C containing an extra proline residue (purple circle). Given the distribution of products, it is unsurprising that the HPLC trace is broad.

The conjugation of 16-amino acid peptide, D, with 1 was followed using HPLC by monitoring the consumption of 1 and the appearance of new species. The reaction products were purified by membrane dialysis. MALDI-TOF mass spectrometry of the isolated product showed peaks

corresponding to 1-D₂, 1-D₃, and 1-D₄. The broadness of the mass spectrum is consistent with both the use of impure peptides and the complications arising from salts of this highly charged peptide. The ¹H-NMR spectrum—albeit broad—showed neither maleimide nor maleic acid signals present in the reaction mixture as might be expected from the perceived low conversion rate. Although thiol addition should be faster than that the addition of any other functional group or solvent, steric congestion could retard this desired reaction and afford opportunities for intermolecular reaction of maleimides with amino acid side chains like that of lysine. The extent to which these reaction can occur as a function of lysine residue position will be assessed in a future study. Using ¹H-NMR, a comparison of peak areas derived from the aromatic phenylalanine peaks and methylene of maleimide suggests that the product distribution is centered at 1-D_{3.5}, a more favorable ratio than what might be expected from casual inspection of the mass spectrum.

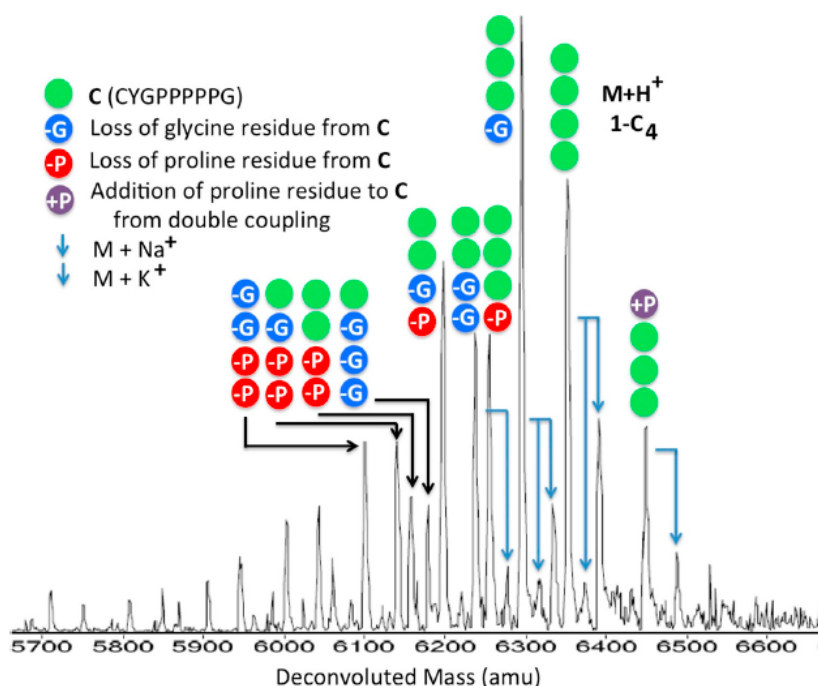


Figure 4. Tentative assignment of the products of conjugation of peptide C with 1 to yield 1-C₄.

3. Experimental Section

3.1. General Synthetic Procedures

All chemicals were purchased from Aldrich (St. Louis, MO, USA) and Acros (Fair Lawn, NJ, USA) and used without further purification. All solvents were ACS grade and used without further purification. HPLC was carried out using an Agilent Technologies (Santa Clara, CA, USA) 1260 Infinity system and an Agilent Technologies 1260 Infinity DAD detector. NMR spectra were recorded on a Bruker Ascend 400 MHz spectrometer (Billerica, MA, USA) in CDCl₃, CD₃OD, or D₂O. All ESI mass spectral analyses were carried out by an Agilent Technologies 6224 TOF LC/MS system.

The chromatographic system used to measure sample purity consisted of a degasser (Agilent G1379B, Palo Alto, CA, USA), capillary pump (Agilent G1312B), micro well-plate auto sampler (Agilent G1367D), eclipse XDB-C18 column (4.6 mm i.d. × 150 mm, 5 μm, Agilent), and a diode array detector (Agilent G1316B). The mobile phase consisted of water/acetonitrile (A/B, HPLC grade, 0.1% (*w/v*) trifluoroacetic acid) at a flow rate of 0.8 mL/min. The elution gradient was 10% MeCN for 5 min, ramp to 90% MeCN in 30 min, and ramp down to 10% MeCN in 15 min. The sample volume injected 5 μL at a concentration 0.1 mg/mL with HPLC-grade MeCN, and detected at 214 nm.

3.2. Synthesis of Dendrimer 1

To a solution of **6** (31 mg, 0.014 mmol) and DIPEA (20 μ L, 0.115 mmol) in dioxane (1 mL), a solution of maleimide–NHS ester (32 mg, 0.115 mmol) in dichloromethane (1 mL) was added at RT then stirred for 48 h. The reaction was concentrated and purified by column chromatography (DCM:MeOH = 97:5 \rightarrow DCM:MeOH = 8:2) to give **1** (Figure 5) as a foam (28 mg, 69%). $^1\text{H-NMR}$ (400 MHz, CDCl_3): δ 6.71 (s, 8H), 3.64–3.32 (m, 120H, $\text{CH}_2\text{OCH}_2\text{CH}_2\text{OCH}_2\text{CH}_2\text{OCH}_2$, $\text{C}_3\text{N}_3\text{-NHCH}_2\text{CH}_2\text{CH}_2\text{O}$, DOTA- $\text{CONHCH}_2\text{CH}_2\text{CH}_2\text{O}$, $\text{CONHCH}_2\text{CH}_2\text{CH}_2\text{N}$, Maleimide- $\text{CONHCH}_2\text{CH}_2\text{CH}_2\text{O}$), 2.15 (t, $J = 7.2$, 8H), 1.92 (dt, $J = 14$, 7.2, 8H), 1.89–1.77 (br m, 28H, $\text{OCH}_2\text{CH}_2\text{CH}_2$); $^{13}\text{C-NMR}$ (100 MHz, CDCl_3) δ 171.7, 170.9, 134.1, 70.5 ($\text{OCH}_2\text{CH}_2\text{O}$), 70.2 ($\text{OCH}_2\text{CH}_2\text{O}$), 70.1 ($\text{OCH}_2\text{CH}_2\text{O}$), 69.8, 69.2 ($\text{CH}_2\text{CH}_2\text{CH}_2\text{O}$), 38.1 ($\text{NH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{O}$), 37.7 ($\text{CH}_2\text{CH}_2\text{CH}_2\text{O}$), 37.3 (maleimide), 33.6 (maleimide), 29.5 ($\text{NH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{O}$), 29.0 ($\text{NH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{O}$), 24.8 (maleimide); MS (ESI-TOF) calcd. for $\text{C}_{127}\text{H}_{214}\text{N}_{31}\text{O}_{40}$ 2813.5664, found 2813.5751 $[\text{M} + \text{H}]^+$. Spectra appear in the Supplementary Materials: Figures S1–S5.

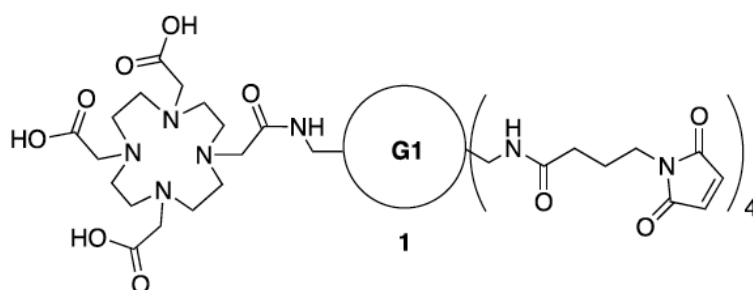


Figure 5. Intermediate 1.

3.3. Synthesis of Intermediate S1—Maleic Acid Amide of 1

A solution of **1** (8 mg) in water (1 mL) was adjusted to pH 7–8 with 1 N NaOH at RT then stirred for 18 h. The reaction mixture was lyophilized to give Intermediate S1 (Figure 6). $^1\text{H-NMR}$ (400 MHz, D_2O): δ 6.23 (d, $J = 12.4$, 4H), 5.83 (d, $J = 12.4$, 4H), 3.55–3.12 (m, 120H, $\text{CH}_2\text{OCH}_2\text{CH}_2\text{OCH}_2\text{CH}_2\text{OCH}_2$, $\text{C}_3\text{N}_3\text{-NHCH}_2\text{CH}_2\text{CH}_2\text{O}$, DOTA- $\text{CONHCH}_2\text{CH}_2\text{CH}_2\text{O}$, $\text{CONHCH}_2\text{CH}_2\text{CH}_2\text{N}$, Maleimide- $\text{CONHCH}_2\text{CH}_2\text{CH}_2\text{O}$), 2.18 (t, $J = 7.4$, 8H), 1.89–1.77 (br m, 36H, $\text{OCH}_2\text{CH}_2\text{CH}_2$, Maleimide); $^{13}\text{C-NMR}$ (100 MHz, D_2O) δ 180.0 (DOTA-acid), 179.9 (DOTA-acid), 175.7 (Maleimide- $\text{CONHCH}_2\text{CH}_2\text{CH}_2\text{O}$), 174.3 (DOTA-amide), 173.2 (DOTA-acid), 168.1 (conjugate amide), 165.3 (triazine, conjugate acid), 135.7, 124.9, 69.7, 69.6, 69.5, 69.4, 68.6, 68.4, 38.5, 37.4, 37.3, 36.4, 33.1, 28.9, 28.3, 24.9 (maleimide); MS (ESI-TOF) calcd. for $\text{C}_{127}\text{H}_{222}\text{N}_{31}\text{O}_{44}$ 2885.6087, found 2885.6806 $[\text{M} + \text{H}]^+$. Spectra appear in the Supplementary Materials: Figures S6–S9.

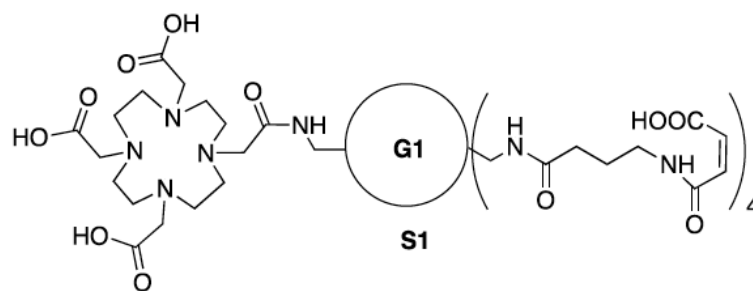


Figure 6. Intermediate S1.

3.4. Synthesis of Intermediate 4

To a solution of **2** (194 mg, 0.09 mmol) and **3** (52 mg, 0.09 mmol) in acetonitrile (8 mL), HBTU (34 mg, 0.09 mmol) was added followed by triethylamine (25 μ L, 0.18 mmol) at RT. The reaction

was stirred for 18 h. The reaction was diluted with 30 mL dichloromethane and washed 0.1 N HCl (30 mL), 10% NaHCO₃ (30 mL), brine (30 mL), then dried over MgSO₄, and concentrated. The crude product was purified by column chromatography (DCM:MeOH = 97:5 → DCM:MeOH = 85:15) to give **4** (Figure 7) as a foam (105 mg, 43%). ¹H-NMR (400 MHz, CDCl₃): δ 3.68–3.47 (m, 104H, CH₂OCH₂CH₂OCH₂CH₂OCH₂, C₃N₃-NHCH₂CH₂CH₂O, Dota-CONHCH₂CH₂CH₂O), 3.22–3.18 (br m, 8H, BocNHCH₂), 1.84–1.72 (m, 28H, OCH₂CH₂CH₂), 1.46 (s, 9H), 1.45 (s, 9H), 1.42 (s, 45H); ¹³C-NMR (100 MHz, CDCl₃) δ 176.6 (DOTA-OCO^tBu), 174.4 (DOTA-OCO^tBu), 172.3 (DOTA-OCO^tBu), not found (C₃N₃), 156.0 (NHCO^tBu), 81.7 (DOTA-OC(CH₃)₃), 78.7 (C(CH₃)₃), 70.47 (OCH₂CH₂O), 70.21 (OCH₂CH₂O), 70.17 (OCH₂CH₂O), 70.10 (OCH₂CH₂O), 69.42 (CH₂CH₂CH₂O), 69.02 (CH₂CH₂CH₂O), 57.5 (DOTA), 56.2 (DOTA), 55.6 (DOTA), 53.5 (DOTA), 41.9 (NH₂CH₂CH₂CH₂O), 38.5 (CH₂CH₂CH₂O), 38.4 (CH₂CH₂CH₂O), 29.6 (NH₂CH₂CH₂CH₂O), 29.3 (NH₂CH₂CH₂CH₂O), 28.4 (C(CH₃)₃), 27.94 (DOTA-OC(CH₃)₃), 27.87 (DOTA-OC(CH₃)₃); MS (ESI-TOF) calcd. for C₁₂₇H₂₄₂N₂₇O₃₆ 2721.7936, found 2721.8117 [M + H]⁺. Spectra appear in the Supplementary Materials: Figures S11–S13.

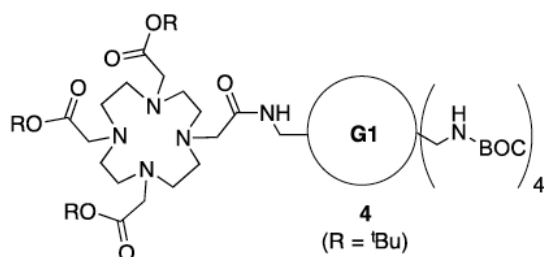


Figure 7. Intermediate 4.

3.5. Synthesis of Intermediate 5

To a solution of **4** (59 mg, 0.022 mmol) in methanol (2 mL), 6 N HCl (aq) (2 mL) was added at RT then stirred for 8 h. The reaction was diluted with ethyl acetate (10 mL) and water (2 mL). Water layer was basifying with 5 N NaOH (aq) then the desired compound was extracted with dichloromethane (10 mL × 8). Combined organic layers was dried over MgSO₄, and concentrated to give **5** (40.5 mg of as a clear oil; Figure 8). ¹H-NMR (400 MHz, CDCl₃): δ 3.63–3.42 (m, 104H, CH₂OCH₂CH₂OCH₂CH₂OCH₂, C₃N₃-NHCH₂CH₂CH₂O, Dota-CONHCH₂CH₂CH₂O), 3.10 (br s, 8H, NH₂CH₂), 1.96–1.82 (br m, 28H, OCH₂CH₂CH₂), 1.45 (s, 27H); ¹³C-NMR (100 MHz, CDCl₃) δ 172.5 (DOTA-OCO^tBu), 171.5 (DOTA-OCO^tBu), 165.8 (C₃N₃), 81.8 (DOTA-OC(CH₃)₃), 81.7 (Dota-OC(CH₃)₃), 70.54 (OCH₂CH₂O), 70.42 (OCH₂CH₂O), 70.19 (OCH₂CH₂O), 70.11 (OCH₂CH₂O), 69.95 (OCH₂CH₂O), 69.40 (CH₂CH₂CH₂O), 69.27 (CH₂CH₂CH₂O), 56.1 (Dota), 55.7 (Dota), 55.6 (DOTA), 39.0 (NH₂CH₂CH₂CH₂O), 38.1 (CH₂CH₂CH₂O), 29.7 (NH₂CH₂CH₂CH₂O), 29.5 (NH₂CH₂CH₂CH₂O), 29.1 (NH₂CH₂CH₂CH₂O), 28.10 (DOTA-OC(CH₃)₃), 27.98 (DOTA-OC(CH₃)₃); MS (ESI-TOF) calcd for C₁₀₇H₂₁₀N₂₇O₂₈ 2321.5839, found 2321.6677 [M + H]⁺. Spectra appear in the Supplementary Materials: Figures S14–S16.

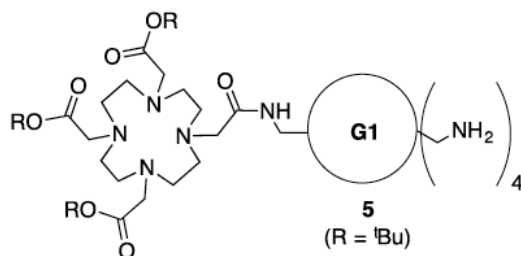


Figure 8. Intermediate 5.

3.6. Synthesis of Intermediate—Generation 3 Dendrimer with DOTA

A solution of **5** (79 mg, 0.034 mmol) and **M** (Macromonomer [24]) (542 mg, 0.27), and DIPEA (71 mL, 0.41 mmol) in THF (3 mL), dioxane (1 mL), *i*-propanol (70%) (0.5 mL) was stirred at 75 °C in a capped vessel for 4 d. The solution was evaporated under vacuum. The residue was dissolved in dichloromethane, washed with brine, dried over MgSO_4 , and concentrated. The crude product was purified by column chromatography. The crude product was purified by column chromatography (DCM:MeOH = 97:5 \rightarrow DCM:MeOH = 85:15) to give **S3** (Figure 9) as a foam (92 mg, 27%). $^1\text{H-NMR}$ (400 MHz, CDCl_3): δ 3.65–3.42 (m, 464H, $\text{CH}_2\text{OCH}_2\text{CH}_2\text{OCH}_2\text{CH}_2\text{OCH}_2$, $\text{C}_3\text{N}_3\text{-NHCH}_2\text{CH}_2\text{CH}_2\text{O}$, $\text{Dota-CONHCH}_2\text{CH}_2\text{CH}_2\text{O}$), 3.22–3.19 (br m, 32H, BocNHCH_2), 1.81–1.72 (br m, 124H, $\text{OCH}_2\text{CH}_2\text{CH}_2$), 1.47 (s, 9H), 1.45 (s, 9H), 1.44 (s, 9H), 1.42 (s, 144H); $^{13}\text{C-NMR}$ (100 MHz, CDCl_3) δ not found (DOTA- OCO^tBu), not found (DOTA- OCO^tBu), not found (DOTA- OCO^tBu), not found (C_3N_3), 156.1 (NHCO^tBu), 81.85 (DOTA- $\text{OC}(\text{CH}_3)_3$), 81.79 (DOTA- $\text{OC}(\text{CH}_3)_3$), 78.8 ($\text{C}(\text{CH}_3)_3$), 70.56 ($\text{OCH}_2\text{CH}_2\text{O}$), 70.28 ($\text{OCH}_2\text{CH}_2\text{O}$), 70.23 ($\text{OCH}_2\text{CH}_2\text{O}$), 70.19 ($\text{OCH}_2\text{CH}_2\text{O}$), 69.52 ($\text{CH}_2\text{CH}_2\text{CH}_2\text{O}$), 69.22 ($\text{CH}_2\text{CH}_2\text{CH}_2\text{O}$), 69.17 ($\text{CH}_2\text{CH}_2\text{CH}_2\text{O}$), not found (DOTA), not found (DOTA), not found (DOTA), 53.4 (DOTA), 41.8 ($\text{NH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{O}$), 38.5 ($\text{CH}_2\text{CH}_2\text{CH}_2\text{O}$), 38.2 ($\text{CH}_2\text{CH}_2\text{CH}_2\text{O}$), 29.67 ($\text{NH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{O}$), 29.60 ($\text{NH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{O}$), 29.54 ($\text{NH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{O}$), 29.33 ($\text{NH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{O}$), 28.5 ($\text{C}(\text{CH}_3)_3$), 28.01 (DOTA- $\text{OC}(\text{CH}_3)_3$), 27.94 (Dota- $\text{OC}(\text{CH}_3)_3$); MS (ESI-TOF) calcd for $\text{C}_{463}\text{H}_{878}\text{N}_{111}\text{O}_{132}$ 10106.5403, found 10114.2078 $[\text{M} + \text{H}]^+$. Spectra appear in the Supplementary Materials: Figures S17–S19.

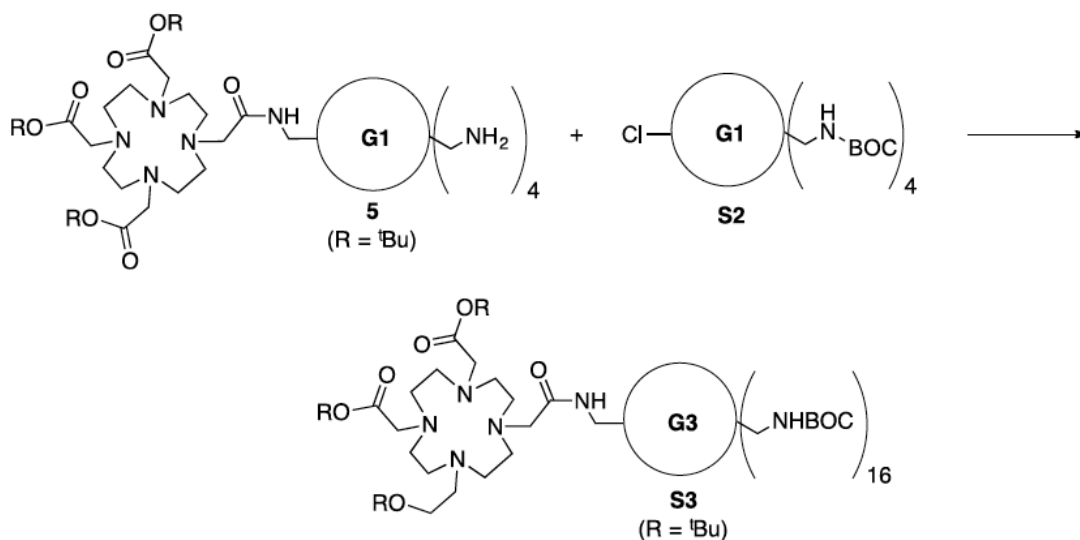


Figure 9. Synthesis of Intermediate S3.

3.7. Synthesis of Intermediate 6

To a solution of **5** (68 mg, 0.029 mmol) in dichloromethane (2 mL), trifluoroacetic acid (2 mL) was added at RT then stirred for 20 h. Subsequently the reaction mixture was evaporated under vacuum. The residue was decanted with diethyl ether (5 mL \times 3), and dried under vacuum to give **6** (Figure 10) as a white powder (quantitative). $^1\text{H-NMR}$ (400 MHz, CD_3OD): δ 3.55–3.26 (m, 104H, $\text{CH}_2\text{OCH}_2\text{CH}_2\text{OCH}_2\text{CH}_2\text{OCH}_2$, $\text{C}_3\text{N}_3\text{-NHCH}_2\text{CH}_2\text{CH}_2\text{O}$, $\text{DOTA-CONHCH}_2\text{CH}_2\text{CH}_2\text{O}$), 2.99 (br t, $J = 6.2$, 8H, NH_2CH_2), 1.85–1.76 (br m, 28H, $\text{OCH}_2\text{CH}_2\text{CH}_2$); $^{13}\text{C-NMR}$ (100 MHz, CD_3OD) δ 164.8, 157.4, 156.4, 71.6, 71.53, 71.50, 71.33, 71.23, 71.21, 70.23, 69.91, 69.76, 69.73, 39.87, 39.64, 39.44, 39.35, 39.27, 39.19, 38.09, 38.03, 30.86, 30.45, 30.35, 30.19, 30.12; MS (ESI-TOF) calcd. for $\text{C}_{95}\text{H}_{186}\text{N}_{27}\text{O}_{28}$ 2153.3961, found 2153.4295 $[\text{M} + \text{H}]^+$. Spectra appear in the Supplementary Materials: Figures S20–S22.

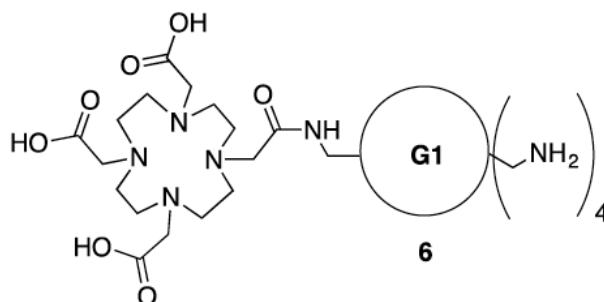
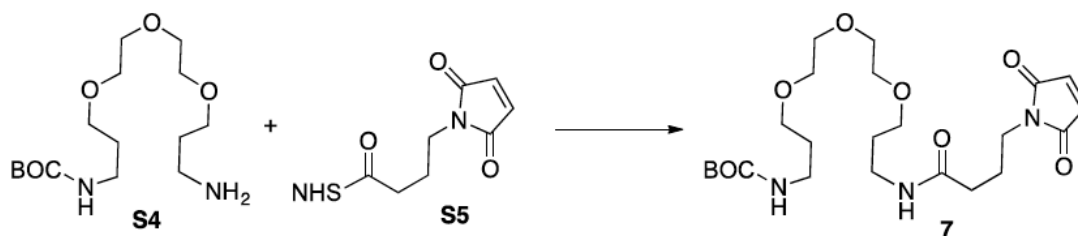


Figure 10. Intermediate 6.

3.8. Synthesis of Model 7

To a solution of commercially-available maleimide succinimide ester **S5** (17 mg, 0.06 mmol) in dichloromethane (3.0 mL), a solution of monoBoc amine **S4** (16 mg, 0.05 mmol) in dichloromethane (1.0 mL) was added at RT. The solution was stirred for 3h at RT and evaporated under vacuum. The crude product was purified by column chromatography (DCM:MeOH = 99:1 → DCM:MeOH = 97:3) to give **7** (Figure 11) as a solid (21 mg, 86%). $^1\text{H-NMR}$ (400 MHz, CDCl_3): δ 6.71 (s, 2H, n), 3.67–3.53 (m, 14H, c, d, e, f, g, h, m), 3.36 (q, J = 6.4, 2H, j), 3.23 (q, J = 6.4, 2H, a), 2.16 (t, J = 6.4, 2H, k), 1.97–1.94 (m, 2H, l), 1.82–1.75 (m, 4H, b, i), 1.43 (s, 9H, Boc) $^{13}\text{C-NMR}$ (100 MHz, CDCl_3) δ 171.6, 170.8, 156.0, 134.1, 78.9, 70.5, 70.4, 70.2, 70.1, 69.8, 69.5, 38.4, 37.8, 37.2, 33.6, 29.6, 28.9, 28.4, 24.7; MS (ESI-TOF) calcd. for $\text{C}_{23}\text{H}_{40}\text{N}_3\text{O}_8$ 486.2815, found 486.4100 $[\text{M} + \text{H}]^+$. Spectra appear in the Supplementary Materials: Figures S23–S25.

Figure 11. Synthesis of **7**.

3.9. Synthesis of Conjugate 7-A

To a solution of **1** (35 mg, 0.07 mmol) in dioxane (1.0 mL) a solution of cysteine **A** (28 mg, 0.079) in dichloromethane (1.0 mL) was added at RT. The solution was stirred for 48h at room temperature and evaporated under vacuum. The crude product was purified by column chromatography (DCM = 100 → DCM:MeOH = 95:5) to give **7-A** (Figure 12) as a oil (37 mg, 61%). $^1\text{H-NMR}$ (400 MHz, CDCl_3): δ 7.76 (d, J = 8.0, 2H, FMOC), 7.61 (t, J = 6.0, 2H, FMOC), 7.42–7.28 (m, 4H, FMOC), 5.00 (br s, 1H, NH), 4.71–4.68 (m, 1H, p), 4.49–4.41 (m, 2H, FMOC), 4.24 (t, J = 6.8, 1H, FMOC), 3.83–3.75 (m, 1H, n), 3.80 (s, 3H, q), 3.65–3.51 (m, 14H, c, d, e, f, g, h, m), 3.46 (dd, J = 14, 6.0, 1H, o), 3.37–3.31 (m, 2H, j), 3.23–3.19 (m, 2H, a), 3.15–3.03 (m, 2H, n', o), 2.50–2.39 (m, 1H, n'), 2.15 (t, J = 7.2, 2H, k), 1.99–1.90 (m, 2H, l), 1.80–1.75 (m, 4H, b, i), 1.43 (s, 9H, BOC) $^{13}\text{C-NMR}$ (100 MHz, CDCl_3) δ 176.7, 174.5, 174.3, 171.5 (ester), 170.9 (amide), 156.0 (Boc), 155.9 (FMOC), 143.8, 143.7, 143.6, 141.3, 127.7, 127.1, 127.0, 125.1, 125.0, 119.98, 119.94, 70.4, 70.1, 70.0, 69.7, 69.4, 67.1, 52.9, 52.8, 47.1, 39.6, 38.6, 38.5, 38.4, 37.7, 36.0, 35.6, 33.5, 33.4, 29.6, 28.9, 28.4, 23.6, 23.55; MS (ESI-TOF) calcd. for $\text{C}_{42}\text{H}_{59}\text{N}_4\text{O}_{12}\text{S}$ 843.3850, found 843.5872 $[\text{M} + \text{H}]^+$. Spectra appear in the Supplementary Materials: Figures S26–S28.

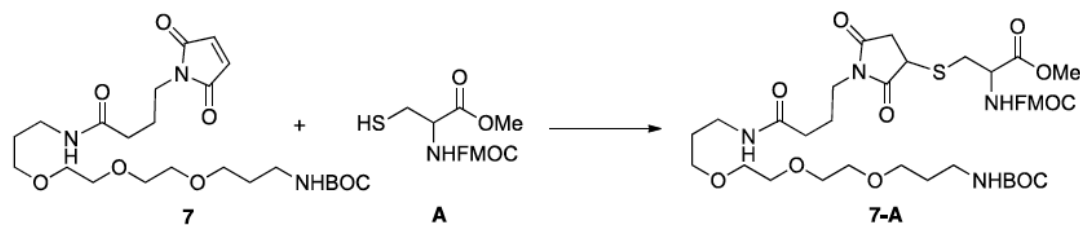


Figure 12. Synthesis of 7-A.

3.10. Synthesis of Cystine (S6)

To a solution of A (59 mg, 0.165 mmol) in dichloromethane (4.0 mL), solid iodine (10 mg, 0.082 mmol) was added at RT. The solution was stirred for 2 h at room temperature and evaporated under vacuum. The crude product was purified by column chromatography (Ethyl Acetate: Hexanes = 1:9 → Ethyl Acetate: Hexanes = 3:7) to give S6 (Figure 13) as white powder (46 mg, 39%). ¹H-NMR (400 MHz, CDCl₃): δ 7.74 (d, *J* = 7.2, 2H, FMOC), 7.59–7.57 (br s, 2H, FMOC), 7.39–7.27 (m, 4H, FMOC), 5.77 (br d, *J* = 7, 1H, NH), 4.70–4.65 (m, 1H), 4.4–4.39 (m, 2H, FMOC), 4.20 (t, *J* = 7.0, 1H, FMOC), 3.74 (s, 3H), 3.17 (d, *J* = 4.8, 2H); ¹³C-NMR (100 MHz, CDCl₃) δ 170.9, 155.7, 143.8, 143.7, 141.3, 127.8, 127.1, 125.1, 120.0, 67.3, 53.3, 52.9, 47.1, 41.1; MS (ESI-TOF) calcd. for C₃₈H₃₇N₂O₈S₂ 713.83898, found 713.859 [M + H]⁺. Spectra appear in the Supplementary Materials: Figures S29–S34.

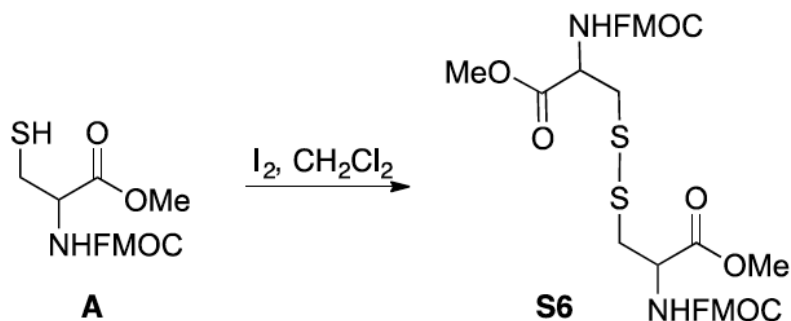


Figure 13. Synthesis of S6.

3.11. Synthesis of Conjugate 7-B

To a solution of 7 (12 mg, 0.025 mmol) in dioxane (1.0 mL), a solution of B (18 mg, 0.033) in dichloromethane (1.0 mL) was added at RT. The solution was stirred for 48 h at room temperature and evaporated under vacuum. The crude product was purified by column chromatography (DCM = 100 → DCM:MeOH = 95:3) to give 7-B (Figure 14) as an oil (16.5 mg, 64%). ¹H-NMR (400 MHz, CDCl₃): δ 7.75 (t, *J* = 7.6, 2H), 7.58 (d, *J* = 7.1, 2H), 7.52 (t, *J* = 8.5, 1H), 7.42–7.25 (m, 6H), 7.14–7.03 (m, 2H), 4.98–4.84 (m, 2H), 4.69–4.44 (m, 1H), 4.44–4.34 (m, 2H), 4.23–4.17 (m, 1H), 4.04 (br s, 1H), 3.59–3.45 (m, 18H), 3.40–2.80 (m, 9H), 2.47–2.41 (m, 1H), 2.23–2.17 (m, 2H), 2.00–1.86 (m, 2H), 1.79–1.75 (m, 4H), 1.43 (s, 9H) ¹³C-NMR (100 MHz, CDCl₃) δ 178.1, 177.5, 174.6, 174.4, 172.1, 172.0, 171.9, 169.7, 169.6, 156.1, 143.8, 143.7, 141.3, 136.6, 136.4, 127.8, 127.4, 127.3, 127.1, 125.1, 124.1, 121.8, 121.7, 120.0, 119.96, 119.91, 119.2, 119.1, 118.4, 118.3, 111.5, 108.6, 108.2, 79.0, 70.5, 70.2, 70.1, 69.8, 69.4, 67.2, 67.0, 53.4, 52.9, 52.7, 52.5, 52.4, 47.1, 47.06, 40.8, 39.4, 38.8, 38.4, 38.2, 37.9, 36.7, 35.9, 33.3, 33.2, 28.5, 27.4, 23.4, 23.3; MS (ESI-TOF) calcd for C₅₃H₆₉N₆O₁₃S 1029.4643, found 1029.6996 [M + H]⁺. Spectra appear in the Supplementary Materials: Figures S35–S38.

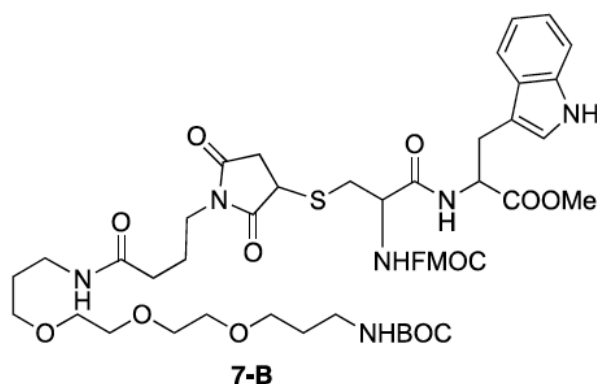
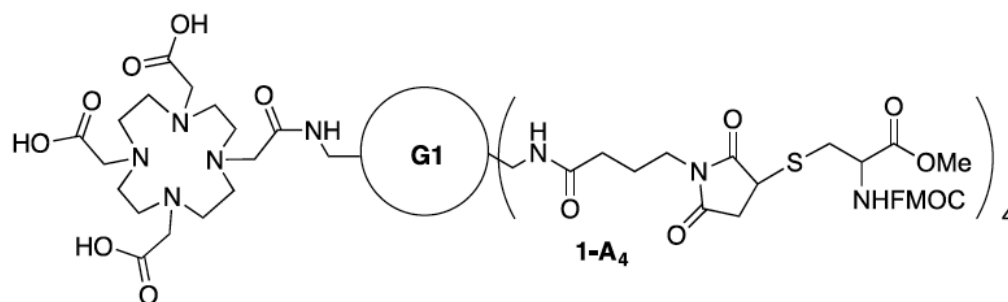


Figure 14. Intermediate 7-B.

3.12. Synthesis of Conjugate 1-A₄

To a solution of **1** (26 mg, 0.009 mmol) in dioxane (1.0 mL) a solution of **A** (25 mg, 0.074) in dichloromethane (1.0 mL) was added at RT. The solution was stirred for 48 h at room temperature and evaporated under vacuum. The crude product was purified by column chromatography (DCM = 100 → DCM:MeOH = 9:1) to give **1-A₄** (Figure 15) as an oil (25 mg, 65%). ¹H-NMR (400 MHz, CDCl₃): δ 7.75 (d, *J* = 7.5, 8H, Fmoc), 7.61 (t, *J* = 7.2, 8H, Fmoc), 7.41–7.29 (m, 16H, Fmoc), 4.68 (br s, 4H, p), 4.44–4.40 (m, 8H, Fmoc), 4.23 (t, *J* = 7.2, 4H, Fmoc), 3.81–3.70 (m, 4H, n), 3.78 (s, 3H, OCH₃), 3.69–3.10 (m, 128H, a, c, d, e, f, g, h, j, m, n', o), 2.48–2.38 (m, 4H, n'), 2.14 (t, *J* = 6.5, 8H, k), 1.94–1.65 (m, 36H, b, i, l); ¹³C-NMR (100 MHz, CDCl₃) δ 177.0, 176.7, 174.6, 174.4, 171.7, 171.0, 156.0, 143.8, 143.7, 141.3, 127.8, 127.1, 125.2, 11.98, 70.55, 70.46, 70.2, 70.0, 69.7, 69.2, 67.1, 54.2, 53.3, 52.9, 52.8, 47.1, 39.7, 38.7, 38.6, 38.2, 37.7, 36.1, 35.7, 34.4, 33.1, 33.52, 33.46, 29.6, 29.3, 29.0, 23.7, 23.6; MS (ESI-TOF) calcd. for C₂₀₃H₂₉₀N₃₅O₅₆S₄ 4241.9804, found 4243.7824 [M + H]⁺. Spectra appear in the Supplementary Materials: Figures S39–S44.

Figure 15. Dendrimers 1-A₄.

3.13. Synthesis of Conjugate 1-B₄

To a solution of **1** (11 mg, 0.0025 mmol) in dioxane (1.0 mL) a solution of **B** (17 mg, 0.031 mmol) in dichloromethane (1.0 mL) was added at RT. The solution was stirred for 48h at room temperature and evaporated under vacuum. The crude product was purified by column chromatography (DCM = 100 → DCM:MeOH = 9:1) to give **1-B₄** (Figure 16) as an oil (10.5 mg, 54%). ¹H-NMR (400 MHz, CDCl₃): δ 7.75–7.00 (m, 52H), 4.89–4.01 (m, 20H, p, q, Fmoc), 3.46–2.95 (m, 152H, a, c, d, e, f, g, h, j, m, n, o, r, OMe), 2.88–2.81 (m, 4H, n'), 2.37–2.31 (m, 4H, n'), 2.28–2.17 (m, 8H, k), 1.97–1.59 (m, 36H, b, i, l); ¹³C-NMR (100 MHz, CDCl₃) δ 177.9, 177.5, 174.6, 174.5, 172.0, 172.0, 169.9, 156.2, 143.8, 143.7, 141.3, 136.6, 136.4, 127.8, 127.4, 127.1, 125.2, 124.1, 121.6, 120.0, 119.94, 119.1, 118.3, 118.2, 111.6, 108.6, 108.3, 70.4, 70.1, 70.0, 69.8, 69.6, 69.1, 67.2, 67.1, 55.1, 53.5, 53.3, 53.0, 52.8, 52.43, 52.38, 47.1, 40.6, 39.4, 38.7, 38.1, 37.9, 37.7, 36.3, 35.9, 33.3, 33.2, 29.7, 29.3, 29.0, 27.4, 23.5, 22.7; MS (ESI-TOF) calcd.

for $C_{247}H_{330}N_{42}O_{60}S_4$ 4986.2976, found 4989.1225 $[M + H]^+$. Spectra appear in the Supplementary Materials: Figures S45–S49.

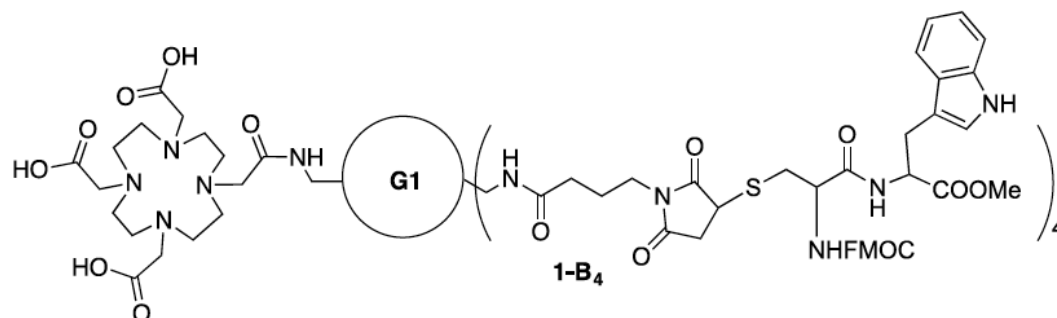


Figure 16. Dendrimer 1-B₄.

3.14. Synthesis of Conjugate 1-C₄

To a solution of maleimide **1** (9.8 mg, 0.0034 mmol) in dioxane (0.5 mL), a solution of CYGPPPPPG C (25 mg, 0.0279 mmol) in water (0.5 mL) was added, followed by addition of DIPEA (10 μ L, 0.056 mmol) at RT. The solution was stirred for 18 h at room temperature and then evaporated under vacuum. The crude product was diluted with deionized water (1.5 mL) and excess amount of peptide was removed from the dendron by centrifugal filtration (3000 MW cut off, 4000 rpm). The residue was washed with deionized water (2 mL) five times and lyophilized to give **1-C₄** (Figure 17) as a solid (14 mg, 63%). $^1\text{H-NMR}$ (400 MHz, D_2O): δ 7.19–7.06 (m, 8H, x), 6.89–6.73 (m, 8H, y), 4.64–4.57 (m, 20H, t), 4.37–4.34 (m, 4H, q), 4.26–4.22 (m, 4H, p), 3.99–3.86 (m, 8H, s), 3.85–3.68 (m, 32H, n, s, w), 3.65–2.81 (m, 156H, a, c, d, e, f, g, h, m, n', o, r, w), 2.59–2.51 (m, 4H, n'), 2.31–2.15 (m, 28H, k, u), 2.05–1.65 (m, 96H, b, i, l, u, v); $^{13}\text{C-NMR}$ (100 MHz, D_2O) δ 178.8, 178.7, 176.8, 174.8, 174.7, 174.1, 173.2, 173.1, 171.7, 171.4, 170.85, 170.7, 169.1, 167.1, 166.9, 162.2, 161.9, 154.0, 153.9, 129.8, 129.75, 127.0, 126.8, 117.0, 114.6, 114.1, 71.3, 68.9, 68.65, 67.8, 67.6, 67.5, 65.8, 61.7, 60.2, 59.7, 58.5, 57.8, 55.7, 55.0, 54.7, 54.6, 51.7, 50.9, 50.0, 47.5, 47.4, 46.9, 46.8, 46.2, 42.0, 41.9, 40.8, 37.8, 37.0, 35.7, 35.4, 34.8, 32.1, 28.5, 27.6, 27.3, 27.1, 23.9, 23.5, 22.2; MS (ESI-TOF) calcd. for $C_{291}H_{442}N_{67}O_{84}S_4$ 6347.1257, found 6295.1574 $[M + H]^+$. Spectra appear in the Supplementary Materials: Figures S50–S55.

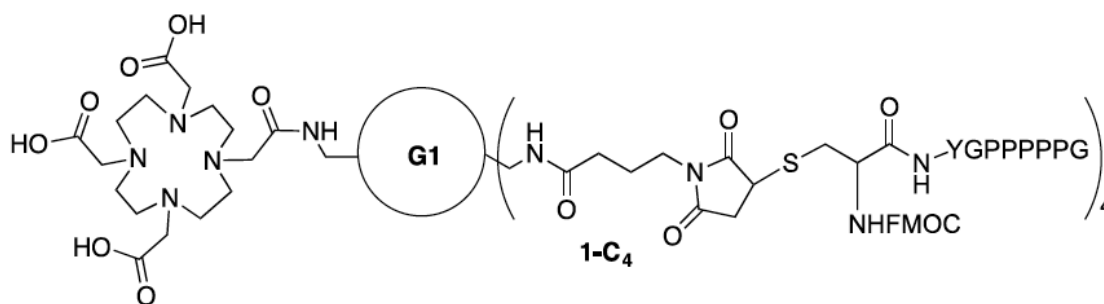


Figure 17. Dendrimer 1-C₄.

3.15. Synthesis of Conjugate 1-D₄

To a solution of **1** (9.1 mg, 0.0032 mmol) in water (0.5 mL), a solution of CGFQLRQPPLVPSRKGE, D (51 mg, 0.026 mmol) in water (0.5 mL) was added followed by addition of 0.1 N NaOH (adjust pH ~ 7–8). The solution was stirred for 18 h at RT before diluting with deionized water (1.0 mL). Excess peptide was removed by centrifugal filtration (3000 MW cut off, 4000 rpm). The residue was washed with deionized water (2 mL) five times and lyophilized to give **1-D₄** (Figure 18) as a solid (19.7 mg,

57%). $^1\text{H-NMR}$ (400 MHz, D_2O): δ 7.25–7.13 (m, 20H, 2), 4.61–4.49 (m, 8H, r, x), 4.39–4.22 (m, 52H, p, s(2), t(2), u(2), v(3), w, y, z), 3.95–2.90 (m, 224H, a, c, d, e, f, g, h, j, m, n, n', o, q(3), 1, 10(2), 11(3), 16, 20), 2.58–2.52 (m, 4H, n'), 2.33–2.13 (m, 44H, k, 4(2), 13(3), 22), 2.13–1.34 (m, 168H, b, i, l, 3(2), 5(2), 6(2), 8(2), 9(2), 12(3), 13(3), 14, 17, 18, 19, 21), 0.92–0.78 (m, 72H, 7(2), 15) $^{13}\text{C-NMR}$ (100 MHz, D_2O) δ 181.0, 179.4, 179.2, 177.7, 176.2, 174.8, 174.3, 174.1, 173.8, 173.3, 173.2, 173.0, 172.4, 171.8, 171.6, 171.2, 170.7, 170.4, 169.8, 156.7, 136.1, 129.2, 128.7, 127.2, 69.7, 69.4, 68.5, 68.4, 61.0, 60.5, 60.0, 58.8, 56.8, 56.4, 55.8, 55.5, 55.0, 53.9, 53.5, 53.2, 53.0, 52.7, 52.5, 50.8, 48.3, 47.9, 47.7, 43.5, 42.5, 40.5, 39.7, 39.1, 38.5, 37.7, 37.1, 36.5, 33.4, 32.9, 31.0, 30.7, 30.2, 30.1, 29.5, 29.3, 28.6, 28.4, 28.2, 27.8, 27.0, 26.2, 24.7, 24.4, 23.0, 22.2, 22.0, 21.9, 21.1, 21.0, 18.3, 17.6; MS (MALDI-TOF) calcd. for $\text{C}_{471}\text{H}_{777}\text{N}_{139}\text{O}_{136}\text{S}_4$ 10684., found 10662. Spectra appear in the Supplementary Materials: Figures S56–S60.

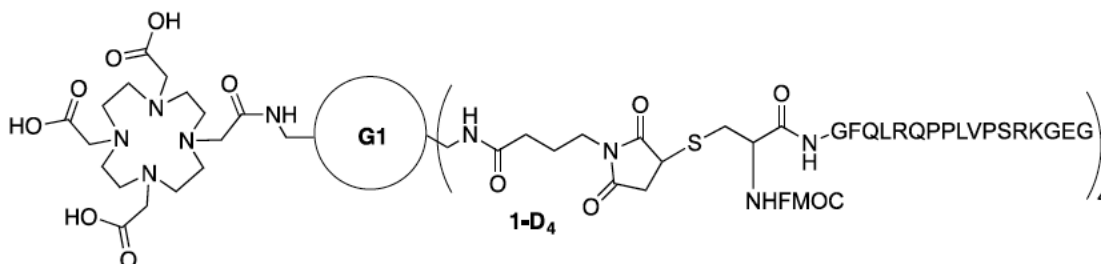


Figure 18. Dendrimer 1- D_4 .

4. Conclusions

In conclusion, **1** represents a valuable platform for the pursuit of theranostic applications. Mass spectrometry provides evidence that, upon metallation with gadolinium, **1-Gd** can be reacted with **A** to yield **1-A₄-Gd**. This construct and related conjugates will be described in due course. While poly(maleimides) are commercially available (Toronto Research Chemicals), the cost and lack of a chelating-group for imaging applications represent limitations overcome by **1**. Both the synthetic and conjugation chemistry described here are straightforward and can be affected with a minimum of effort. However, the extent to which these reactions proceed is dependent on the size and composition of the thiol. Tetravalent displays of large peptides have proven difficult to push to completion. Critically, conjugation can be accomplished in a range of solvents—from organic solvents to aqueous solutions—to incorporate a range of thiol-containing species. The value of this conjugation strategy—maleimide and thiol—over alternatives has been reported: Wangler *et al.* showed that thiol-maleimide couplings proceed more effectively than oxime formation or copper-catalyzed cycloadditions [26]. Finally, we note that, when the flexible dendritic domains are fully extended, **1** places ligands in the corners of a rectangular array measuring ~ 2.5 nm by ~ 4.2 nm.

Supplementary Materials: The following are available online at: <http://www.mdpi.com/1420-3049/21/3/335/s1>; ESI-TOF MS, $^1\text{H-NMR}$, $^{13}\text{C-NMR}$ and Stability test.

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Author Contributions: C.S.L. and E.E.S. conceived and designed the experiments; C.S.L. performed a majority of the experiments; K.J. assisted in the preparation of peptides and conjugation reactions. All contributed to the preparation of the paper.

Conflicts of Interest: The authors declare no conflict of interest.

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Sample Availability: Samples of the compounds are available from the authors.



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Article

Thermoregulated Coacervation, Metal-Encapsulation and Nanoparticle Synthesis in Novel Triazine Dendrimers

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Abstract: The synthesis and solubility behaviors of four generation five (G5) triazine dendrimers are studied. While the underivatized cationic dendrimer is soluble in water, the acetylated and propanoylated derivatives undergo coacervation in water upon increasing temperature. Occurring around room temperature, this behavior is related to a liquid-liquid phase transition with a lower critical solution temperature (LCST) and is explained by differences in composition, notably, the hydrophobic nature of the terminal groups. Interestingly, the water solubility of the acetylated dendrimer is affected by the addition of selected metal ions. Titrating solutions of acetylated dendrimer at temperatures below the LCST with gold or palladium ions promoted precipitation, but platinum, iridium, and copper did not. Gold nanoparticles having diameters of 2.5 ± 0.8 nm can be obtained from solutions of the acetylated dendrimer at concentrations of gold less than that required to induce precipitation by treating the solution with sodium borohydride.

Keywords: dendrimer; triazine; LLPS; LCST; thermoresponsive; nanoparticle, gold

1. Introduction

The temperature-induced, reversible coacervation of macromolecules in solution is a result of liquid-liquid phase separation (LLPS) [1]. Depending on the chemical nature of macromolecule and solvent, LLPS may be induced either by decreasing or increasing temperature. The corresponding temperature-composition phase diagrams will exhibit either an upper critical solution temperature (UCST) or a lower critical solution temperature (LCST), respectively.

This phase transition has been extensively investigated for solutions of linear polymers due to their importance for mixture thermodynamics, separation technologies, self-assembly processes, catalysis and the preparation of thermoresponsive materials [1–5]. However, corresponding studies on dendrimer solutions are scarce [6], which is surprising given the significant overlap between the scopes of dendrimers and LLPS applications. In the case of catalysis, a thermoregulated formation of coacervates of dendrimers could be employed to separate these nanoreactors from the reaction products. In the case of extraction, LLPS could be used to separate the molecules sequestered by the host dendrimers from solution with applications to purification and drug loading. Finally, the coupling of coacervation with chemical crosslinking could be applied to produce crosslinked coacervates with high guest loading capacity, relevant to drug delivery applications.

Our own interest rests in triazine dendrimers [7]. Recent interest in metal nanoparticles led to the serendipitous discovery of dendrimers displaying LLPS behaviors. Specifically, two of these systems undergo reversible opacification upon increasing temperature, characteristic of the LCST type of behavior. To our knowledge, no previous LLPS study has been reported for solutions of triazine dendrimers.

2. Results and Discussion

2.1. Design and Synthesis

The compounds examined in this study derive from **1** (Figure 1), a generation 5 triazine dendrimer composed of triazines linked by 4,7,10-trioxodecanediamine and piperazine [8–10]. Like **1**, dendrimers **2**, **3**, and **4** are generation 5 dendrimers with 96 end groups and molecular weights of approximately 40 kDa. The compound numbers (**2**, **3**, **4**) conveniently reflect the number of carbons in their acyl groups (acetyl, propanoyl, isobutanoyl).

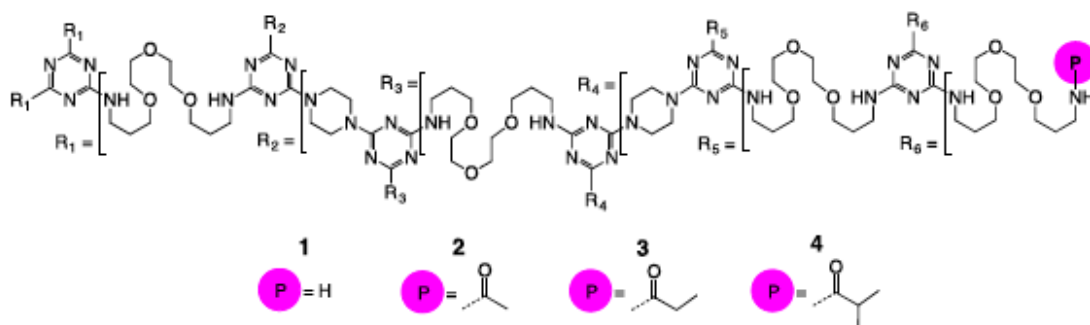


Figure 1. The molecules used in this study.

Generation 5 dendrimers were chosen because this generation shows an onset of globular structure with high degrees of porosity and diameters measuring 6 nm [10]. This size was considered ideal for the creation of dendrimer-encapsulated nanoparticles, the initial impetus for this work [11–24]. The choice of linkers reflects a balance of needs for reactivity during synthesis (piperazine groups) [25,26] and water solubility (4,7,10-trioxodecanediamine) [8–10,27]. Additionally, polyethyleneglycol tethers have been shown to influence Au nanoparticle formation and stability [19,20].

The acetylated dendrimer, **2**, was the first target based on literature precedent. Wang *et al.* have shown that acetylated PAMAM dendrimers lead to the formation of dendrimer-encapsulated nanoparticles that are more biocompatible [22]. Pietsch has shown that acetylation leads to better control over nanoparticle size and shape [23].

To arrive at **2**, progenitor **1** was acetylated with acetic anhydride. Indeed, to arrive at **3** and **4**, **1** was subjected to the commercially available acid anhydrides as well. The preparation of **1** relied on condensing two published molecules, **5** and **6** (Figure 2) to yield **7**. Both **5** and **6** are available by a rapid, microwave and macromonomer-mediate synthesis [8,9]. In addition to providing water solubility, these building blocks were perceived to yield dendrimers that were both large and flexible with pores envisioned to support nanoparticle growth. The reaction is facilitated by the presentation of secondary amines (piperazine) on **5** that show higher reactivity than primary amines [25,26] for monochlorotriazines like **6**. Upon isolation of **7**, a BOC-derivative displaying no water solubility, **1** can be obtained by treatment with acid.

During the course of manipulating **2**, we observed an LCST in water. Manipulating the LCST of a system is commonly done by affecting the hydrophobic/hydrophilic balance. In polyacrylamides, for example, the LCST of a polymer comprising *N*-isopropyl groups can be increased by introducing *N*-ethyl groups or decreased by introducing isobutyl groups [28]. To probe the impact that the acyl group has on LCST, the acetylated dendrimer, **2**, was compared with those presenting propanoyl

groups, 3, and isobutanoyl groups, 4. Like 1, 4 was insoluble in water at room temperature, precluding further analysis.

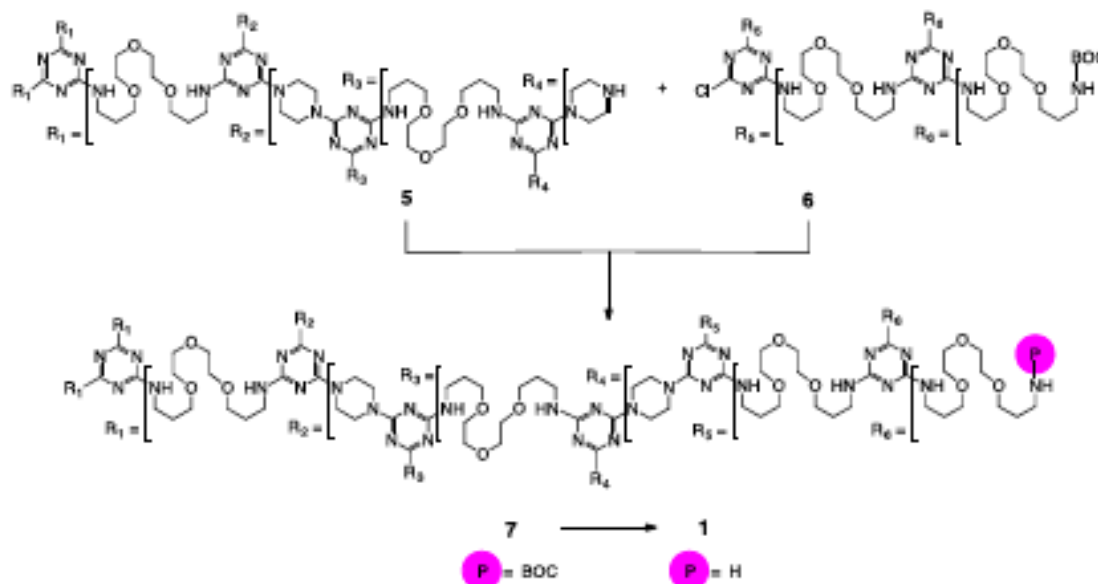


Figure 2. The synthesis of 1 relies on the use of 5 and 6 [12].

2.2. Liquid-Liquid Phase Separation (LLPS) Temperatures

LLPS temperatures, T_{ph} , were determined using the turbidity method described in the Experimental Section. The temperature-induced opacification of aqueous solutions of 2 and 3 at 4 mg/mL are shown in Figure 3. We obtain $T_{ph} = 28^\circ\text{C}$ for 2 and $T_{ph} = 20^\circ\text{C}$ for 3.

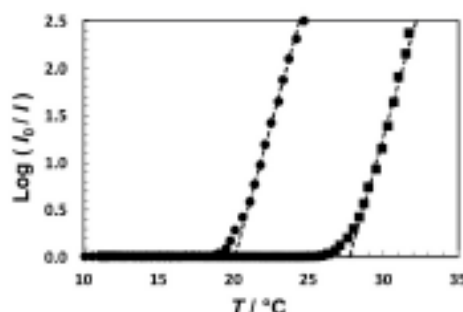


Figure 3. Temperature-turbidity profiles for aqueous solutions of 2 (squares) and 3 (circles). The dendrimer concentration is 4 mg/mL in both cases. The dashed lines, which are linear fits through the data with $\text{Log}(I_0/I) > 0.5$, show the values of $T_{ph} = 20^\circ\text{C}$ (3) and $T_{ph} = 28^\circ\text{C}$ (2) extrapolated at $\text{Log}(I_0/I) = 0$.

LCST behavior is observed for aqueous solutions of polyethylene glycol (PEG) [29]. At room temperature, water is a good solvent for this polymer due to the formation of hydrogen bonds between the PEG ethoxy groups and water molecules. As temperature increases, water becomes a poorer solvent for PEG, thereby leading to LLPS [30]. In our case, PEG-like linkers are used between triazines to enhance water solubility of these dendrimers at room temperature. The observed LCST type of behavior is attributed to the PEG-like domains of our triazine dendrimers. As temperature increases, the hydration of the PEG-like linkers decreases [31], and the solubility of triazine dendrimers is expected to reduce giving rise to LLPS. Similar desolvation behavior of the PEG-like domains has been observed in computational models of these and other dendrimers [32].

The actual location of the LLPS temperature is expected to strongly depend on the chemical nature of the dendrimer terminal groups. That the value of T_{ph} in the case of **3** is lower than that in the case of **2** correlates with the higher hydrophobicity of the propanoyl group compared to that of the acetyl group. Furthermore, our analysis suggests that the LLPS of aqueous solutions of **4** should be located at lower temperatures, consistent with the observed poor solubility of **4** in water. In summary, our findings show that the modifications of the terminal groups of these triazine dendrimers can be used to modulate the LLPS temperature around room temperature.

2.3. Influence of Metal Ions on Solubility of **2**

Dendrimer **2** was chosen for hosting nanoparticles because the LCST was sufficiently high to facilitate their synthesis. However, upon adding approximately 100 mole equivalents of gold in the form of $\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$ to solutions of **2**, a precipitate formed. While we recognize that the equivalence point is close to the number of end groups, additional studies will be required to ascertain the mechanistic basis for this coincidence. A similar behavior was seen with additions of Na_2PdCl_4 . In contrast, titrated additions of K_2PtCl_4 , $\text{IrCl}_3 \cdot x\text{H}_2\text{O}$, or $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ did not promote precipitation. The selectivity observed could be useful, as the separation of gold or palladium from copper is of some interest in the recycling of microelectronic waste streams [33–36]. Simple sequestration strategies are also attracting attention [37,38]. Dendrimers are largely unexplored in this capacity, but the scalable synthesis of triazines makes such opportunities of interest.

2.4. Formation of Nanoparticles

At low molar ratios of Au^{3+} : Dendrimer (approximately 60:1), soluble dendrimer-encapsulated nanoparticles could be prepared by the addition of sodium borohydride, a reagent required to generate Au at room temperature. Figure 4 summarizes the results of these experiments. The mean diameters of these particles was 2.55 ± 0.84 nm. The particles are red in solution and UV-Vis spectroscopy reveals an absorption maximum at 510 nm, consistent with the well-known surface plasmon of gold. Preliminary experiments show that nanoparticles of palladium, platinum, iridium and copper are also accessible, but these studies are preliminary and will be reported in due course. For gold nanoparticles, we note that not only is the particle size reasonably homogeneous, but the spacing between the particles is very similar and close to 6 nm, the diameter of the dendrimer. That is, the micrograph is consistent with a single gold nanoparticle encapsulated within a single globular macromolecule that sterically separates it from its neighbor. Additional experiments will be required to bear out this hypothesis.

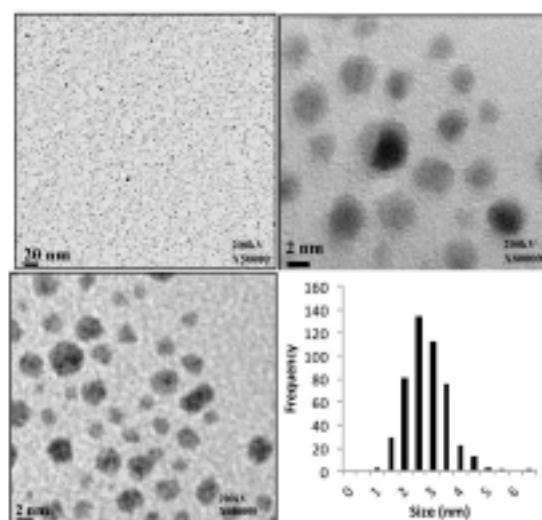


Figure 4. A TEM micrographs at three magnifications of dendrimer-encapsulated gold nanoparticles derived from **2** and the distribution of their sizes.

3. Materials and Methods

3.1. General Experimental

All reagents were used as received. Methanol, dichloromethane, dioxane, diethylether, acetic anhydride, HAuCl_4 , D_2O , CDCl_3 (Sigma-Aldrich, St. Louis, MO, USA); triethylamine (TEA), DIPEA, Na_2PdCl_4 , K_2PtCl_4 , $\text{IrCl}_3 \cdot x \text{H}_2\text{O}$ (Pressure Chemical, Pittsburgh, PA, USA). Microwave reactions were carried on using a CEM SP Discover microwave (CEM Corporation, Matthews, NC, USA). NMR experiments were conducted on a Bruker 400 Ascend spectrometer (Bruker, Billerica, MA, USA); UV-Vis spectra were obtained on an Agilent 8453 (Agilent, Santa Clara, CA, USA) were recorded at room temperature in water 18 m Ω . The NMR data listed shows the theoretical number of protons expected for the molecule reported. Error prevents an accurate assessment of the true numbers, but integration of the spectra corroborate this expectation.

3.2. Preparation of 1

A solution of 7 (0.2605 g, 4 μmol) in dioxane (6 mL) was mixed with HCl conc. (3 mL) and heated 3 min at 60 $^\circ\text{C}$ using dynamic mode. Afterwards, solvent was evaporated under vacuum and residue dissolved in water; pH was adjusted to 12 using 5 M NaOH (aq). The product was extracted with dichloromethane and, after solvent evaporation, deprotected dendrimer was obtained as a white thick oil (0.2069 g, quantitative yield). ^1H -NMR (400 MHz, D_2O) δ 3.77–3.11 (m, 2592H, $\text{CH}_2\text{OCH}_2\text{CH}_2\text{OCH}_2\text{CH}_2\text{OCH}_2$, $\text{C}_3\text{N}_3\text{-NHCH}_2\text{CH}_2\text{CH}_2\text{O}$, $\text{C}_3\text{N}_3\text{-NCH}_2\text{CH}_2\text{N}$), δ 2.74–2.92 (br, 192H, NH_2CH_2), δ 1.86–1.59 (br, 636H, $\text{OCH}_2\text{CH}_2\text{CH}_2\text{NH}$); ^{13}C -NMR (100 MHz, CDCl_3) δ 166.06 (C_3N_3), δ 165.14 (C_3N_3), δ 70.57 ($\text{OCH}_2\text{CH}_2\text{O}$), δ 70.16 (two lines, $\text{OCH}_2\text{CH}_2\text{O}$), δ 69.42 ($\text{NHCH}_2\text{CH}_2\text{CH}_2\text{O}$), δ 69.28 ($\text{NHCH}_2\text{CH}_2\text{CH}_2\text{O}$), δ 43.01 ($\text{NCH}_2\text{CH}_2\text{N}$), δ 39.50 ($\text{NHCH}_2\text{CH}_2\text{CH}_2\text{O}$), δ 38.08 ($\text{NHCH}_2\text{CH}_2\text{CH}_2\text{O}$), δ 33.12 ($\text{NHCH}_2\text{CH}_2\text{CH}_2\text{O}$), δ 29.68 ($\text{NHCH}_2\text{CH}_2\text{CH}_2\text{O}$); MS (MALDI-TOF) calcd for $\text{C}_{1992}\text{H}_{3834}\text{N}_{660}\text{O}_{477}$ 44639.60, found 40742.54. See the Supplementary Materials (Figures S4–S6) for the NMR and MS data of 1.

3.3. Preparation of 2

A mixture of 1 (0.0917 g, 2 μmol), acetic anhydride (93 μL , 985 μmol) and triethylamine (165 μL , 1183 μmol) in methanol (13 mL) was stirred overnight at room temperature. Afterwards, solvent was evaporated and residue dissolved in water; then, impurities were filtered off using ultracentrifugation (30 min, 14,000 rpm) and product was washed two more times with pure water. After lyophilization, a pale yellow sticky solid was obtained (0.0497 g, 50% yield). ^1H -NMR (400 MHz, D_2O) δ 3.12–3.60 (m, 2592H, $\text{CH}_2\text{OCH}_2\text{CH}_2\text{OCH}_2\text{CH}_2\text{OCH}_2$, $\text{C}_3\text{N}_3\text{-NHCH}_2\text{CH}_2\text{CH}_2\text{O}$, $\text{C}_3\text{N}_3\text{-NCH}_2\text{CH}_2\text{N}$), δ 3.05–3.12 (t, 192H AcNHCH_2), δ 1.57–1.77 (br, 636H, $\text{OCH}_2\text{CH}_2\text{CH}_2\text{NH}$), δ 1.84 (s, 288H, COCH_3); ^{13}C -NMR (100 MHz, D_2O) δ 173.44 (CO), δ 164.76 (br, C_3N_3), δ 69.70 ($\text{OCH}_2\text{CH}_2\text{O}$), δ 69.42 (two lines, $\text{OCH}_2\text{CH}_2\text{O}$), δ 68.64 ($\text{NHCH}_2\text{CH}_2\text{CH}_2\text{O}$), δ 68.45 ($\text{NHCH}_2\text{CH}_2\text{CH}_2\text{O}$), δ 42.88 ($\text{NCH}_2\text{CH}_2\text{N}$), δ 37.50 ($\text{NHCH}_2\text{CH}_2\text{CH}_2\text{O}$), δ 36.49 ($\text{NHCH}_2\text{CH}_2\text{CH}_2\text{O}$), δ 28.96 ($\text{NHCH}_2\text{CH}_2\text{CH}_2\text{O}$), δ 28.35 ($\text{NHCH}_2\text{CH}_2\text{CH}_2\text{O}$), δ 21.89 ($\text{C}(\text{CO})$); MS (MALDI-TOF) calcd for $\text{C}_{2184}\text{H}_{4026}\text{N}_{660}\text{O}_{573}$ 48672.62, found 40652.96. See the Supplementary Materials (Figures S7–S9) for the NMR and MS data of 2.

3.4. Preparation of 3

A mixture of 1 (0.039 g, 0.873 μmol), propionic anhydride (53 μL , 419 μmol) and triethylamine (70 μL , 503 μmol) in methanol (10 mL) was stirred overnight at room temperature. Afterwards, solvent was evaporated and residue dissolved in water; then, impurities were filtered off using ultracentrifugation (30 min, 14,000 rpm) and product was washed two more times with pure water. After lyophilization, a pale yellow sticky solid was obtained (0.009 g, 21% yield). ^1H -NMR (400 MHz, D_2O) δ 3.10–3.60 (m, 2592H, $\text{CH}_2\text{OCH}_2\text{CH}_2\text{OCH}_2\text{CH}_2\text{OCH}_2$, $\text{C}_3\text{N}_3\text{-NHCH}_2\text{CH}_2\text{CH}_2\text{O}$, $\text{C}_3\text{N}_3\text{-NCH}_2\text{CH}_2\text{N}$), δ 2.10–2.04 (m, 192H $\text{CH}_3\text{CH}_2\text{CONHCH}_2$), δ 1.45–1.95 (br, 636H, $\text{OCH}_2\text{CH}_2\text{CH}_2\text{NH}$), δ 0.91–0.97 (t, 288H, COCH_2CH_3); ^{13}C -NMR (100 MHz, D_2O)

δ 176.6 (CO), δ 165.2 (br, C_3N_3), δ 69.7 (OCH_2CH_2O), δ 69.4 (two lines, OCH_2CH_2O), δ 68.5 ($NHCH_2CH_2CH_2O$), δ 42.88 (NCH_2CH_2N), δ 37.4 ($NHCH_2CH_2CH_2O$), δ 36.4 ($NHCH_2CH_2CH_2O$), δ 29.1 ($NHCH_2CH_2CH_2O$), δ 29.0 ($NHCH_2CH_2CH_2O$), δ 28.4 (CH_3CH_2CON), δ 9.7 (CH_3CH_2CON); MS (MALDI-TOF) calcd for $C_{2280}H_{4218}N_{660}O_{573}$ 50018.12, found 47376.9. See the Supplementary Materials (Figures S10–S12) for the NMR and MS data of 3.

3.5. Preparation of 4

A mixture of 1 (0.039 g, 0.873 μ mol), isobutyric anhydride (100 μ L, 603 μ mol) and triethylamine (14 μ L, 100 μ mol) in methanol (10 mL) was heated overnight at 40 °C. Afterwards, solvent was evaporated and residue dispersed in water; then, impurities were filtered off using ultracentrifugation (30 min, 14,000 rpm) and product was washed two more times with pure water. After lyophilization, a pale yellow sticky solid was obtained (0.029 g, 65% yield). 1H -NMR (400 MHz, D_2O) δ 3.21–3.60 (m, 2592H, $CH_2OCH_2CH_2OCH_2CH_2OCH_2$, $C_3N_3-NHCH_2CH_2CH_2O$, $C_3N_3-NCH_2CH_2N$), δ 3.05–3.22 (m, 192H $CH_3CH_2CONHCH_2$), δ 2.31–2.38 (m, 96, $CH(CH_3)_2$), δ 1.64–1.73 (br, 636H, $OCH_2CH_2CH_2NH$), δ 0.99–0.97 (d, 576H, $CH(CH_3)_2$); ^{13}C -NMR (100 MHz, D_2O) δ 180.0 (CO), δ 165.2 (br, C_3N_3), δ 69.7 (OCH_2CH_2O), δ 69.5 (OCH_2CH_2O), δ 69.4 (OCH_2CH_2O), δ 68.5 ($NHCH_2CH_2CH_2O$), δ 42.88 (NCH_2CH_2N), δ 38.2 ($NHCH_2CH_2CH_2O$), δ 37.6 ($NHCH_2CH_2CH_2O$), δ 35.1 ($CH(CH_3)_2$), δ 28.7 ($NHCH_2CH_2CH_2O$), δ 18.7 ($CH(CH_3)_2$); MS (MALDI-TOF) calcd for $C_{2376}H_{4506}N_{660}O_{573}$ 51460.37, not found. See the Supplementary Materials (Figures S13 and S14) for the NMR spectra of 4.

3.6. Preparation of 7

Synthesis of 7 was accomplished following an analogous procedure to the one reported for Enciso *et al.* [9]. A solution of 5 (0.0711 g, 9 μ mol) in methanol (0.5 mL) was mixed with another solution of 6 (0.9157 g, 461 μ mol) in dioxane (4 mL), and DIPEA (0.2 mL, 1148 μ mol) was added. Then mixture was heated for 6 h at 95 °C. Solvent was evaporated and product impurities were removed washing the crude several times with diethyl ether. A white solid, 7, was obtained (0.3681 g, 71% yield). 1H -NMR (400 MHz, $CDCl_3$) δ 3.25–3.95 (m, 2592H, $CH_2OCH_2CH_2OCH_2CH_2OCH_2$, $C_3N_3-NHCH_2CH_2CH_2O$, $C_3N_3-NCH_2CH_2N$), δ 3.02–3.25 (br, 192H $BocNHCH_2$), δ 1.54–1.96 (br, 636H, $OCH_2CH_2CH_2NH$), δ 1.38 (s, 864H, $C(CH_3)_3$); ^{13}C -NMR (100 MHz, $CDCl_3$) δ 164.56 (br, C_3N_3), δ 156.04 (CO), δ 78.77 ($C(CH_3)_3$), δ 70.53 (OCH_2CH_2O), δ 70.15 (two lines, OCH_2CH_2O), δ 69.48 ($NHCH_2CH_2CH_2O$), δ 69.15 ($NHCH_2CH_2CH_2O$), δ 43.0 (NCH_2CH_2N), δ 38.24 ($NHCH_2CH_2CH_2O$), δ 38.45 ($NHCH_2CH_2CH_2O$), δ 29.58 ($NHCH_2CH_2CH_2O$), δ 29.46 ($NHCH_2CH_2CH_2O$), δ 28.44 ($C(CH_3)_3$); MS (MALDI-TOF) calcd for $C_{2472}H_{4602}N_{660}O_{669}$ 54244.64, found 50180.07. See the Supplementary Materials (Figures S1–S3) for the NMR and MS data of 7.

3.7. Measurement of the Cloud Point

The phase separation temperature, T_{ph} , was determined by measuring the turbidity of binary dendrimer-water samples as function of temperature. A binary dendrimer-water homogenous sample with a dendrimer concentration of 4 mg/mL was prepared by mixing known amounts of water and dendrimer. The turbidity meter is comprised of a programmable circulating bath (1197P, VWR), a calibrated thermocouple (± 0.1 °C), a homemade optical cell, where the initially-transparent sample (optical path of 0.4 cm) and a thermocouple probe are located. Collimated light from a solid state laser (633 nm, 5 mW, Coherent) passes through the sample and its transmittance is recorded by a photodiode detector coupled with a computer-interfaced optical meter (1835-C Newport). After recording the transmitted intensity of the transparent sample, I_o , the temperature of the bath is changed at a constant rate of ± 0.5 °C/min and the transmitted intensity, I , is recorded as a function of temperature, T . We identify T_{ph} as the temperature at which a sharp decrease in intensity is observed (see Figure 3).

3.8. Titrations

A stock solution of **2** (0.0184 g, 0.37 μmol) in water (3 mL) were prepared. For every complexation experiment, 100 μL of stock solution were diluted with 900 μL of water in a 1 mL quartz cuvette to yield a dendrimer concentration (1.25×10^{-5} M). Aqueous solutions of metal salts (typically at $10 \times$ lower concentration) were freshly prepared and added under stirring using a micropipette; no more than 100 μL were added to the dendrimer solution to avoid dilution effects; mixtures were allowed to equilibrate 10 minutes (Cu, Pt, Ir, and Au) or 15 min (Pd).

3.9. Nanoparticle Synthesis

The same stock solution of metal-dendrimer (**2**) titration experiments was used. For every reduction experiment 100 μL of stock solution were diluted with 900 μL of water in a 1 mL quartz cuvette. Aqueous solutions of metal precursors were freshly prepared and added under stirring using a micropipette (30 μL , 60 metal equivalents). Mixture was left to equilibrate for a period of 15 min before NaBH_4 were added (20 μL , metal to NaBH_4 ratio 1:4) under vigorous stirring. The procedure was carried out at room temperature.

3.10. Electron Microscopy

Samples of dendrimer-encapsulated gold nanoparticles were analyzed using transmission electron microscopy (JEOL Ltd., Tokyo, Japan) on a JEOL JEM-2100 operating at 200 kV. Approximately 50 μL of a given sample was applied to a carbon-coated copper grid and allowed to air dry.

4. Conclusions

Aqueous solutions of some generation 5 triazine dendrimers display LLPS with an LCST type of behavior. The LCST of these materials can be varied with the choice of terminal acyl group: Acetylated dendrimers remain soluble at higher temperatures than propanoylated dendrimers. Curiously, the LCST appears to be affected by the addition of some metal ions, but not others. Indeed, this behavior could translate into strategies for the selective recovery of some precious metal salts like gold and palladium in the presence of less valuable ones such as copper. Gold nanoparticles can still be achieved, however, by performing the reduction at concentrations below which precipitation occurs.

Supplementary Materials: Supplementary materials can be accessed at: <http://www.mdpi.com/1420-3049/21/5/599/s1>.

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Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

The following abbreviations are used in this manuscript:

DEN	Dendrimer-encapsulated nanoparticle
G5	Generation 5
LCST	Lower critical solution temperature
LLPS	Liquid-liquid phase separation
PAMAM	Polyamidoamine
PPI	Polypropyleneimine

NaBH ₄	Sodium tetrahydroborane
TEM	Transmission electron microscopy

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